INDEX NUMBER

# The Journal of LABORATORY and CLINICAL MEDICINE

VOL. 43 JUNE, 1954 NO. 6

*The Official Publication of the Central Society for Clinical Research*

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Vol. 43, No. 6, June, 1954. The Journal of Laboratory and Clinical Medicine is published monthly by The C. V. Mosby Company, 3207 Washington Blvd., St. Louis 3, Mo. Subscription price: United States and its Possessions $12.00 per year; Canada $12.50; Foreign $13.50. Subscriptions to medical students, interns and residents, half-price. Entered as Second Class Matter October 8, 1915, at the Post Office at St. Louis, Missouri, under Act of March 3, 1879. Printed in the U. S. A.

The Journal of Laboratory and Clinical Medicine

VOL. 43 JUNE, 1954 NO. 6

## CLINICAL AND EXPERIMENTAL

# HUMAN CORTICOSTERONE METABOLISM

## IV. PROBABLE PREDOMINANCE OF CORTICOSTERONE IN THE ADRENOCORTICAL SECRETION OF PREMATURE INFANTS

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BECAUSE the urinary excretion of Zimmermann chromogens (17-ketosteroids) is affected by testicular function, Landau and associates[1] have suggested that the determination of urinary Pettenkofer chromogens[2] may be a more valid measure of adrenal function. Wolfson and associates[3] have shown that corticosterone is a precursor of Pettenkofer chromogen but not of Zimmermann chromogen. 17-hydroxysteroids are the major precursors of adrenal 17-ketosteroids, but do not contribute to the Pettenkofer reaction. Table I summarizes the effect of the administration of 17-hydroxycorticoids, corticosterone, and corticotropin to normal adults on the excretion of Zimmermann chromogen and Pettenkofer chromogen. The ratio of the excretion of these substances may reflect the ratio of the secretion of corticosterone and hydrocortisone by the adrenal gland.[3]

Both the Pettenkofer reaction and the Allen reaction[4] were once considered to be almost specific tests for dehydroepiandrosterone (DHA). Using the Allen test, Eagle[5] found that the neutral steroid extracts from the urine of premature infants frequently gave a positive test. This was attributed to increased excretion of DHA. After Wolfson and colleagues[6] discovered that DHA was not the principal steroid contributing to the Pettenkofer reaction

Received for publication Oct. 22, 1953.

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**From The Unit for Clinical Investigation, Department of Internal Medicine, Wayne University College of Medicine, Detroit, Mich. Dr. Wolfson's studies are in part assisted by grants-in-aid from the Armour Laboratories, The Wilson Laboratories, and from the Medical Research and Development Board, Office of the Surgeon-General, Department of the Army, under contract DA-49-007-MD-452.

after corticotropin or corticosterone, the possibility that DHA was not the substance being excreted by premature infants suggested itself. The present study has confirmed this suggestion.

In addition, the effects of the administration of ACTH and cortisone on the excretion of Zimmermann, Allen, and Pettenkofer chromogen by the premature infant has been investigated.

TABLE I. THE EFFECT OF THE ADMINISTRATION OF 17-HYDROXYCORTICOIDS, CORTICOSTERONE, AND CORTICOTROPIN TO NORMAL ADULTS ON THE EXCRETION OF ALLEN AND ZIMMERMANN CHROMOGENS, AND ON THE RATIO BETWEEN THEM

| | ALLEN CHROMOGEN | ZIMMERMANN CHROMOGEN | ALLEN / ZIMMERMANN |
|---|---|---|---|
| 17-Hydroxy-corticoids | No change | Increased | Decreased |
| Corticosterone | Increased | No change | Increased |
| Corticotropin | Increased | Increased | No change |

## MATERIALS AND METHODS

Fourteen premature infants were studied for periods of three or four days' duration. On the first study day, no medication was given. On subsequent days, nine infants received 10 units of long-acting corticotropin (Acthar gel, Armour), and six infants received 25 mg. of cortisone acetate. Both medications were given by subcutaneous injection. (One infant was studied first with long-acting corticotropin and subsequently with cortisone acetate). Each twenty-four-hour urine was collected as previously described.[5] From each urine a crude neutral extract was prepared and tested for Zimmermann chromogen by Bongiovanni's procedure.[7] Allen chromogen was determined by Gardner and Migeon's[8] modification of the original procedure of Allen and associates.[4]

A positive Allen "blue test," consisting of the development of a blue color, is qualitative, and indicates the presence of relatively large amounts of material (previously presumed to be DHA) which absorb light at 600 millimicrons. A negative test does not necessarily mean that blue reacting material is absent, since other chromogens may obscure or modify the visible color. Both positive and negative tests may be used quantitatively, however, by utilizing Allen's technique of "skimming the peak" of the spectral absorption curve at 600 millimicrons,[9] provided that the spectral absorption curve of other chromogens is linear in the range between 580 and 620 millimicrons. In the course of this study it was found that the spectral absorption curve in this area of chromogens other than DHA could not be assumed to be linear. Actually the four Pettenkofer chromogens, in addition to DHA, must each have a peak in the neighborhood of 600 millimicrons. Therefore the quantitative measurements of Allen chromogen (expressed as milligram equivalents of DHA) cannot be considered to be more than approximations. It is our impression that this technique of measurement grossly underestimates the real value when only small amounts of Allen chromogen are present.[10]

All urine extracts which gave a positive test were pooled. The crude neutral extract was chromatographed by means of Savard's ligroin-propylene glycol system[11] on Munktell 20-B paper. A DHA standard was run simultaneously on each sheet. Color development was accomplished by passing the sheet through a chloroform solution saturated with antimony trichloride. In previous studies this color reaction has been found to give results exactly paralleling those of the Pettenkofer reaction applied to paper. The antimony trichloride reaction gives more distinct spots and, because the sheets do not promptly disintegrate, they may be scanned quantitatively. Four chromogens are found, and have been named PC-1, PC-2, PC-3, and PC-4 in order of increasing polarity. PC-4 was first detected in this study. Considerations bearing on the possible structure of the Pettenkofer chromogens are presented elsewhere.[12]

RESULTS

1. *Qualitative Nature of the Steroids Excreted by Premature Infants Giving a Fositive Allen Test.*—No significant amount of DHA was found on paper chromatograms prepared from these strongly positive Allen reacting

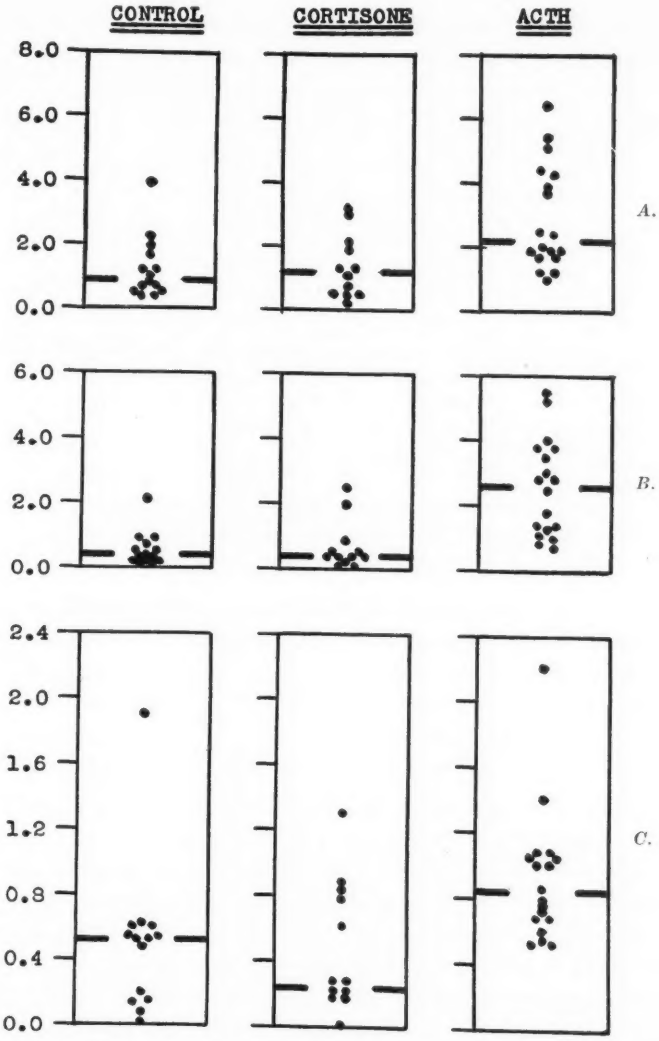


Fig. 1.—Effect of intramuscular cortisone and long-acting ACTH on neutral Zimmermann chromogen and neutral Allen chromogen in the urine of premature infants. (Median values are indicated by heavy horizontal bars.) *A*, Urine Zimmermann chromogen, milligrams per day, as dehydroepiandrosterone. *B*, Urine Allen chromogen, milligrams per day, as dehydroepiandrosterone. *C*, Urine Allen chromogen over urine Zimmermann chromogen.

urine extracts. The three previously known Pettenkofer chromogens were demonstrated by strongly positive spots, and in addition, a moderately intense PC-4 spot, found in the beta-ketonic fraction, developed many hours after the other spots. This steroid has not been seen previously in normal

nonpregnant or pregnant adults, or in adults given corticotropin, cortisone, hydrocortisone, corticosterone, or 11-keto-progesterone. Its very rapid movement suggests that it may be an artifact.

2. *Effect of ACTH and Cortisone on Allen and Zimmermann Chromogens.*—Fig. 1 summarizes the quantitative changes in Zimmermann and Allen chromogens under control conditions and after cortisone and ACTH.

During the control periods, Zimmermann chromogens averaged 0.9 mg. per day, the Allen chromogens averaged 0.4 mg. per day, and the ratio of Allen to Zimmermann chromogen had a mean value of 0.52. This may be compared to normal adult values of 0.20.[13, 14] Qualitatively, the Allen reaction was positive, giving a visibly blue color whenever this ratio was above 0.5, and no blue color was seen when this ratio fell below this value. In all instances, however, there was a definite peak of 600 millimicrons on the spectral absorption curve, enabling quantitative measurement. The incidence of qualitatively positive Allen tests in premature infants has been determined in this and previous studies to be of the order of 41 per cent (thirty-three of eighty-one samples).

During cortisone administration, Zimmermann chromogen increased to median values of 1.2 mg. per day. There was no change in the excretion of Allen chromogen, and median value of the ratio of Allen to Zimmermann chromogen fell to 0.24. These results are entirely consistent with similar studies in adults.

After ACTH, the Zimmermann chromogen increased to mean values of 2.2 mg. per day. The ratio of Allen to Zimmermann chromogen increased to a median value of 0.84. This is in marked contrast to what is found in normal adults where the ratio does not change after ACTH because the Allen and Zimmermann chromogens increase proportionately. Qualitatively, the Allen reaction was positive in every instance.

3. *Effect of ACTH on Pettenkofer and Zimmermann Chromogens.*—After finding Pettenkofer chromogens rather than DHA in chromatograms of Allen positive urines, the Pettenkofer and Zimmermann chromogens were determined in a second study. In a pooled sample of urine from untreated premature infants, the Pettenkofer chromogen was 2.53 mg. per day, the Zimmermann chromogen was 0.90 mg. per day, and the P/Z ratio was 2.81. In a pooled sample of urine obtained from infants treated with ACTH, the Pettenkofer chromogen was 3.43 mg. per day, and the Zimmermann chromogen was 3.96 mg. per day, giving a P/Z ratio of 0.87. Both before and after ACTH, the ratios are exceedingly high. Similar values have been seen only in adults receiving corticosterone.

A comparison of Allen/Zimmermann ratios and Pettenkofer/Zimmermann ratios reveals that they are both much higher in premature infants than in adults. The changes in the ratios after ACTH were in opposite directions, however. This may be explained by our impression that the measurement procedure for Allen chromogen tends to underestimate its value when only small amounts are present.

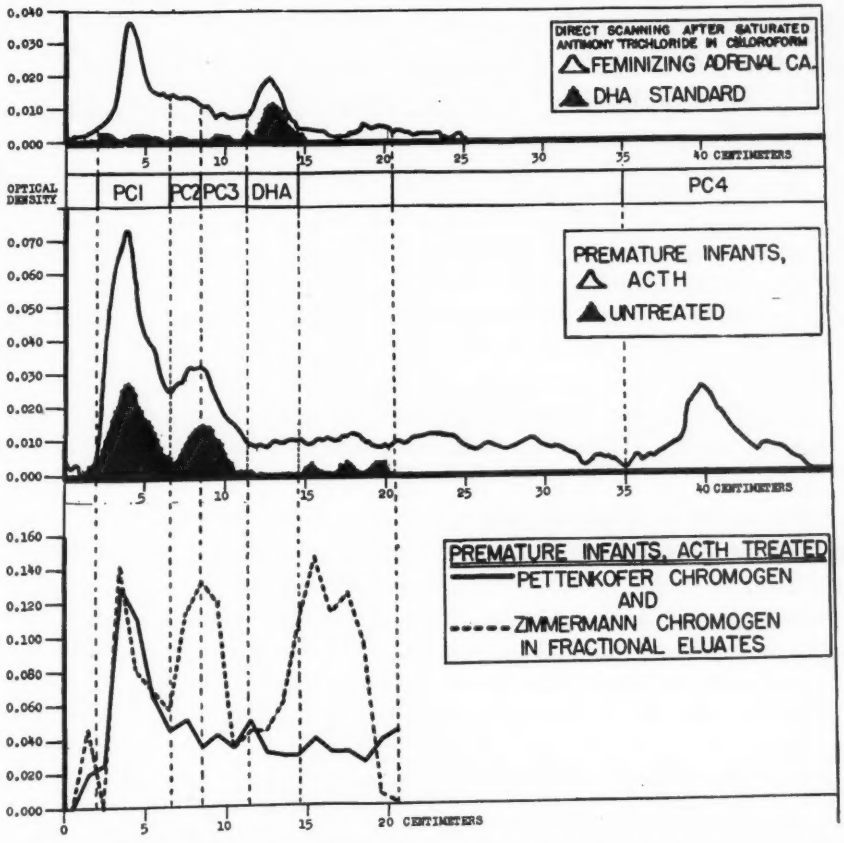


Fig. 2.—Simultaneous paper chromatography of a dehydroepiandrosterone standard and of crude neutral fractions from untreated premature infants, from premature infants receiving corticotropin, and from a patient with metastatic feminizing adrenal carcinoma.

The five cromatograms shown were run on the same sheet of paper. The time of development was deliberately brief to keep PC-4 from overflowing the paper but, for this reason, differentiation of PC-2 from PC-3 is poor in these samples. The upper four samples were treated with the antimony trichloride reagent and then evaluated by direct photometric scanning. The fifth strip, obtained from the corticotropin-treated infants, was examined only for the first 21 cm. of its length (i.e., through the DHA area). The strip was cut into 1 cm. blocks and each block was eluted with methanol. Half of the eluate was used for Pettenkofer chromogen estimation and half for Zimmermann chromogen estimation. The parallelism of the Pettenkofer and antimony trichloride reaction is apparent even by this technique, but less obvious than when the Pettenkofer reaction is developed directly on the paper. It should be noted that the Pettenkofer/Zimmermann ratio in the PC-1 area is much higher than in the DHA region.

The over-all Pettenkofer/Zimmermann ratio of the crude neutral extract was 0.87 for the corticotropin-treated infants, 2.81 for the untreated infants, and 0.94 for the carcinoma patient. It is nevertheless apparent that only the latter sample contained large amounts of dehydroepiandrosterone, even though all three samples were prepared simultaneously and in an identical manner. This finding shows: (1) an extremely high Pettenkofer/Zimmermann ratio is not necessarily accompanied by a high concentration of DHA, and (2) even though strong acid hydrolysis was used in these studies, the technique is entirely satisfactory for the detection of increased amounts of DHA when such amounts actually are present.

The sample from the patient with adrenal carcinoma was supplied by Dr. Grosvenor W. Bissell who will discuss the case fully in a later report. Other interesting aspects of this patient's steroid excretion pattern include a marked elevation of estrogen output, a 17-hydroxy-corticoid output of 49.2 mg. per day, and a 17-desoxy-corticoid output of 83.1 mg. per day. The chromatogram shown in this illustration bears an interesting resemblance to that seen in normal pregnant women, which also shows the predominance of PC-1 and DHA.[12]

Chromatography of these steroids together with the steroids from a patient with feminizing adrenal cortical carcinoma are shown in Fig. 2. The presence of DHA in this latter patient indicates that the methods of hydrolysis, extraction, and chromatography are adequate to detect DHA if it is present along with the Pettenkofer chromogens.

## DISCUSSION

It is not surprising, considering the method of hydrolysis, that only minimal amounts of DHA were found. DHA is notoriously fragile; treatment with heat and strong acid, results in recoveries of less than 40 per cent. In addition, it should be noted that in a number of instances, particularly after the administration of corticotropin, the Allen/Zimmermann ratio exceeded 1.0. Since DHA is used as the standard for the estimation of both Zimmermann and Allen chromogens, the theoretical maximum value would be 1.0 if the Allen chromogen determination actually was specific for DHA. The occurrence of values above 1.0, therefore, requires the presence of steroid that participate in the Allen reaction but do not contribute equally to the color of the Zimmermann reaction.

In these studies it has been shown that Pettenkofer chromogens are present in relatively large quantities in neutral steroid extracts giving a positive Allen test, and that the Pettenkofer/Zimmermann ratio is increased under circumstances where the Allen/Zimmermann ratio is increased. It is recognized, however, that no additional evidence has been offered to substantiate the impression, gained from the literature and a consideration of the factors necessary for both color reactions, that each test actually measures the same steroids. Therefore, the comparison between them may not be strictly valid.

The following hypothesis appears to explain all of the present observations. The adrenal gland of the premature infant secretes corticosterone (Allen chromogen precursors) and hydrocortisone (Zimmermann chromogen precursors) in a ratio which is higher than that of the adult, and hence the urinary Allen/Zimmermann ratio is higher.

A corollary of this hypothesis may well provide an explanation for the low output of 17-ketosteroids in children in whom there is no suggestion of adrenal insufficiency. Corticosterone will provide all necessary life-maintaining activity, and yet will not contribute to 17-ketosteroid excretion. If the hypothesis is expanded to assume that the adrenal gradually alters its corticosterone/hydrocortisone ratio as the child grows and develops, the appearance of 17-ketosteroids during middle childhood (usually called the "adrenarche") is more easily understood. Teleologically, this hypothesis has advantages, for it suggests that adrenal cortical sufficiency during the period of most rapid growth is maintained not by hydrocortisone, but by corticosterone, a considerably less potent growth inhibitor. It is perhaps of some interest that of all mammals so far studied, the ratio of corticosterone to hydrocortisone secretion is highest in the rat, an animal which never stops growing.

And finally this hypothesis suggests that careful evaluation of the relative merits of hydrocortisone and corticotropin as therapeutic agents may be re-

quired in children. If the anti-inflammatory effects of hydrocortisone are desired, it would appear logical to administer it rather than ACTH, where the principal effect might be increased adrenal secretion of corticosterone.

## SUMMARY

Neutral lipid extracts prepared from strong acid-hydrolyzed urine of premature infants contain a much higher amount of Allen "blue test" reactive chromogen and Pettenkofer reactive chromogen relative to the content of Zimmermann chromogen than do extracts from the urine of normal adults. As in adults, cortisone increases the amount of Zimmermann chromogen but not that of Allen chromogen, and corticotropin causes an increase in Allen, Pettenkofer, and Zimmermann chromogen. The very high ratios of Pettenkofer to Zimmermann chromogens has been seen previously only in adults receiving corticosterone.

This has suggested that the adrenal cortex of the premature infant has a higher corticosterone/hydrocortisone secretory ratio than does the adult under basal conditions, and that the infant's adrenal cortex responds to stimulation with a steroid mixture in which corticosterone predominates.

Analysis of the infants' neutral lipid extracts by paper chromatography has given findings consistent with this hypothesis. No significant amount of DHA could be detected in the pooled extracts from all of the Allen positive urines. Instead, the major spot pattern present was similar to that seen in extracts prepared from the urines of adults receiving large amounts of corticosterone. In addition, one new spot, possibly an artifact, was noted.

The hypothesis suggested assumes that hydrocortisone production occurs later than does corticosterone production. This may explain the absence of significant excretion of 17-ketosteroids in early childhood. The changeover from predominant corticosterone secretion to predominant hydrocortisone secretion, rather than an "adrenarche," may be responsible for the beginning of significant 17-ketosteroid excretion in middle childhood.

Since the major effect of corticotropin may be increased adrenal secretion of corticosterone rather than hydrocortisone, it would appear to be preferable to administer hydrocortisone to children rather than corticotropin if the anti-inflammatory effects of hydrocortisone are desired.

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# CLINICAL EXPERIENCE WITH P-SULFONAMIDO-BENZOIC ACID, A CARBONIC ANHYDRASE INHIBITOR, AS A DIURETIC AGENT

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## INTRODUCTION

THE desirability of using an oral diuretic agent in the treatment of edema is apparent to all physicians and in recent years a number of compounds have been investigated in an effort to obviate the necessity of frequent parenteral injections. Among these, drugs which inhibit the action of carbonic anhydrase have been reported to effect diuresis incident to an alteration in the ion-exchange mechanism in the renal tubules.

Although it has been known since 1940 that sulfanilamide is an inhibitor of carbonic anhydrase,[1, 2] it was not until 1949 that a clinical study describing its use in edematous subjects was reported.[3] Other workers have systematically examined a large number of sulfonamide derivatives,[4, 5] and from this group have emerged two compounds which have been studied clinically. The better known of these is Diamox (compound 6063, or 2-acetylamino-1, 3, 4-thiadiazole-5-sulfonamide) which has an in vitro inhibiting power 330 times that of sulfanilamide.[5] The physiological effects of Diamox have been described by Berliner who clearly showed augmentation of sodium, potassium, bicarbonate, and water excretion, associated with abolition of urine titratable acid excretion, when this agent was administered to dogs.[6, 7] In addition, several clinical studies[8-10] have been reported, which showed favorable diuresis when Diamox was administered to edematous patients. The second compound utilized, Dirnate, or p-sulfonamidobenzoic acid, was found by Krebs[4] to be a powerful inhibitor of carbonic anhydrase. Merrill[11] reported that eight of fifteen edematous subjects treated orally with Dirnate responded with a significant natriuresis, although the details of his findings were not presented.

The present study is designed to screen the clinical usefulness of Dirnate as a diuretic agent.

## MATERIALS AND METHODS

All patients were hospitalized white men. Other therapeutic measures (digitalis, bed rest, and so forth) were unaltered during the period of study. With the exception of H. A., the subjects received daily a diet which contained approximately 35 meq. of sodium. They were studied on a metabolic ward, where twenty-four-hour specimens of urine were collected and analyzed for sodium, potassium, chloride, and creatinine. Constancy of urinary creatinine excretion was accepted as evidence of accurate collection techniques. In addition, urine was collected under oil at varying times of the day to determine urine pH (not corrected to body temperature). Venous blood from an antecubital vein was drawn without stasis, made incoagulable with heparin, and the plasma analyzed for sodium, potassium,

From the Veterans' Administration Hospital and Department of Medicine, University of Utah College of Medicine, Salt Lake City, Utah.
Received for publication, Nov. 23, 1953.

TABLE I. ORAL ADMINISTRATION OF DIRNATE

| PATIENT | AGE | DIAGNOSIS | EDEMA | DIRNATE (GM./DAY) | DAYS OF TREATMENT | URINE ELECTROLYTE EXCRETION MEQ./DAY | | | | | WEIGHT CHANGE (POUNDS) | AVERAGE URINE pH (CONTROL) | AVERAGE URINE pH (DIRNATE) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | SODIUM (CONTROL) | SODIUM (DIRNATE) | SODIUM (INCREMENT) | POTASSIUM (CONTROL) | POTASSIUM (DIRNATE) | POTASSIUM (INCREMENT) | | | |
| G. H. | 27 | Nephrosis | ++++ | 8 | 7 | 13.9 | 24.0 | 10.1 | 46.6 | 50.6 | 4.0 | +2 | 6.13 | 6.31 |
| E. T. | 62 | Cirrhosis | Ascites only | 8 | 4 | <1 | <1 | — | 14.7 | 41.3 | 26.6 | +2 | — | — |
| R. P. | 29 | Amyloid | + | 8 | 4 | <1 | <1 | — | 46.3 | 31.9 | −14.4 | −2 | — | — |
| D. C. | 57 | ASHD | + | 8 | 4 | 33.6 | 49.9 | 16.3 | 44.3 | 70.4 | 26.1 | −1 | — | — |
| R. F. | 57 | Cor pulmonale | +++ | 8 | 3 | 2.7 | 3.3 | 0.6 | 25.2 | 50.0 | 24.8 | +2 | 5.63 | 5.25 |
| J. O. | 61 | RHD | ++ | 8 | 4 | 51.4 | 70.2 | 18.8 | 36.5 | 42.5 | 6.0 | −6 | — | — |
| R. Q. | 54 | Cirrhosis | Ascites only | 8 | 3 | <1 | <1 | — | 24.8 | 21.3 | − 3.5 | 0 | — | — |
| H. A. | 57 | ASHD | TR. | 8 | 3 | 167.1 | 154.5 | −12.6 | 81.3 | 104.6 | 23.3 | +1 | — | — |

SYMBOLS: ASHD—Arteriosclerotic heart disease
RHD—Rheumatic heart disease
The conventional 1- to 4- plus grading of edema is used

*The control electrolyte excretion represents the average excretion in milliequivalents per day over a two- to four-day control period, while the electrolyte excretion for the experimental period represents average daily excretion during the entire three- to seven-day period during which Dirnate was administered. Increments in Na and K excretion are expressed as average daily increase in milliequivalents per day during the experimental period.

chloride, and creatinine; blood for pH determination was drawn into a greased syringe, tested immediately, and corrected to body temperature. The weight of the patient was determined daily before breakfast.

The oral studies were conducted over a three- to seven-day period, following a control period equaling in length the experimental period. Dirnate was administered in the form of 0.5 Gm. tablets, the usual daily dose being 8 Gm. given in four divided dosages. A control period was considered satisfactory only if body weight were constant within a range of 1.5 pounds over a period of at least three days. Water was allowed ad libitum.

Acute studies utilizing the agent intravenously were performed with the patient in a fasting, hydrated state. Dirnate was given intravenously in a dose of 20 mg. per kilogram in 250 ml. of 5 per cent dextrose in water over a twenty-minute period, after two or three forty-five-minute control periods during which urine flow was observed to be constant.

Nine patients were given Dirnate orally (six with heart disease, two with cirrhosis, and one with nephrosis) and four patients were given the drug intravenously (three with heart disease and one with nephrosis).

Sodium and potassium were determined with a Beckman flame photometer; pH with a Cambridge Research Model pH Meter; chloride by the method of Schales[13]; $CO_2$ combining power by the method of Van Slyke[14]; and creatinine by the Jaffe reaction.[12]

## RESULTS

*Oral Administration.*—The results of the oral administration of Dirnate to eight patients are shown in Table I. It will be noted that, in general, increments in sodium and potassium excretion were of a low order of magnitude, a finding which correlated well with the failure of these patients to achieve satisfactory weight loss. Indeed, more than one-half of the patients either failed to lose weight or actually gained weight. The three subjects who lost weight lost only an average of about one pound over a three-day period, which is less than the acceptable change in weight during the control period. Three of the patients whose rates of excretion of sodium during the control period were low (less than 1 meq. per day) showed no detectable increase in excretion while taking the drug.

Determinations of plasma sodium, potassium, $CO_2$ combining power, and whole blood pH, showed no significant or consistent changes. The pH of urine voided in the morning before the first dose of Dirnate was invariably below 7.0; sufficient determinations of urine pH were not done during the day to detect any significant trends.

It was regularly found that, when an increase in sodium and potassium excretion did occur, it was maximal during the first and second days of the administration of the drug and that, by the third day, rates of excretion had approached control values.

*Intravenous Administration.*—Three patients were given Dirnate intravenously (Table II). The expected physiological response is shown in graphic form in Fig. 1, patient H. A. This patient had only slight edema at the time of the experiment and was ingesting approximately 100 meq. of sodium daily. The marked increments of urine flow and urine sodium and potassium are readily apparent; urine flow increased nearly eightfold, sodium excretion sixfold, and potassium excretion nearly fourfold. The urine promptly became alkaline

TABLE II. INTRAVENOUS ADMINISTRATION OF DIRNATE*

| PATIENT | AGE | DIAGNOSIS | DIRNATE I.V. (GM.) | PERIOD | URINARY EXCRETION/MIN. VOLUME (ML.) | NA (μEQ.) | K (μEQ.) | URINE pH | PLASMA NA (MEQ./L.) | K (MEQ./L.) | $CO_2$ (MEQ./L.) | pH |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| H. A. | 57 | ASHD | 1.04 | Control | 0.9 | 48.7 | 39.6 | 5.20 | — | — | — | — |
| | | | | Maximum | 7.7 | 300.0 | 169.0 | 7.29 | — | — | — | — |
| G. H. | 27 | Nephrosis | 1.48 | Control | 4.0 | 32.9 | 32.2 | 6.48 | 136 | 3.8 | 21.2 | 7.28 |
| | | | | Maximum | 5.2 | 59.7 | 37.6 | 6.61 | 143 | 3.9 | 22.1 | 7.31 |
| C. M. | 49 | ASHD | 1.78 | Control | 3.0 | 150.0 | 45.0 | 6.71 | 139 | 4.3 | 23.7 | — |
| | | | | Maximum | 6.7 | 452.0 | 103.0 | 7.53 | 139 | 4.2 | 24.4 | — |

*The control value for urine Na, K, and volume is the average excretion during three forty-five-minute control periods; maximum value represents excretion during the first or second forty-five-minute collection period following Dirnate, as maximum excretion rates always occurred during one of these two periods.

and remained so for 135 minutes; only when urine pH dropped below 7.0 did electrolyte excretion approach control values. Of note is the fact that this patient did not lose weight during the twenty-four hours after the experiment; instead, he gained 1.5 pounds. This result illustrates that, although the anticipated diuretic and natriuretic response may be produced, the response is of such a short duration and low magnitude in comparison with the desired total twenty-four-hour excretion that little significant clinical response is obtained.

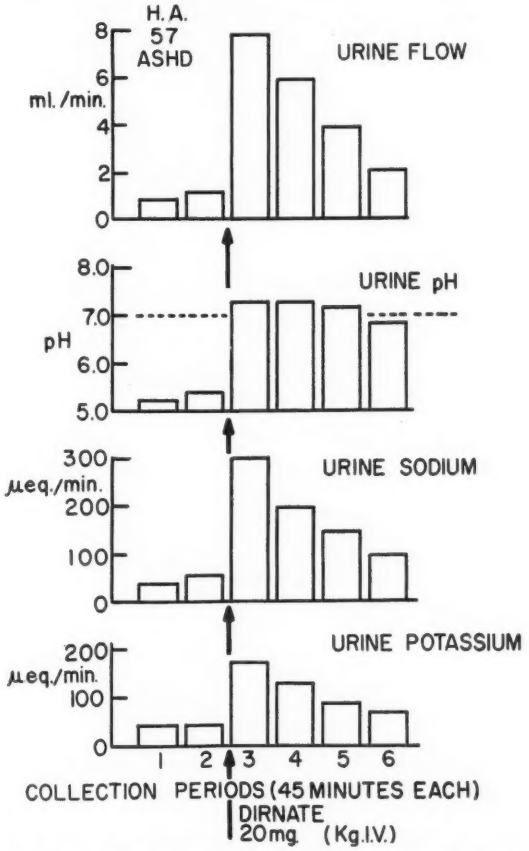


Fig. 1.—Intravenous administration of Dirmate. Details of changes in urine composition and volume are shown.

The essential data on the other two patients are shown in Table II. G. H., a twenty-five-year-old patient with nephrosis, exhibited less than a twofold increase in sodium excretion, and his urinary pH rose only from 6.50 to 6.61. The third patient, C. M., a forty-nine-year-old subject with decompensated arteriosclerotic heart disease, exhibited an intermediate response with prompt alkalinization of the urine but only a slightly more than twofold increase in sodium and potassium excretion.

In two of these experiments, serial determinations of plasma sodium, potassium, and $CO_2$ combining power, as well as of whole blood pH, were made. No significant alteration in these values was observed.

TABLE III. COMPARISON OF MAXIMAL TWENTY-FOUR-HOUR EFFECT IN PATIENTS RECEIVING DIRNATE AND MERCURIAL DIURETICS

| PATIENT | AGE | DIAGNOSIS | DRUG | 24-HOUR URINARY EXCRETION | | | | | WEIGHT CHANGE PER 24 HOURS (POUNDS) |
|---|---|---|---|---|---|---|---|---|---|
| | | | | NA (MEQ.) | K (MEQ.) | Cl (MEQ.) | CREATININE (MG.) | VOLUME (ML.) | |
| R. H. | 57 | RHD | Control | 3.1 | 20.6 | 12.6 | 824 | 900 | — |
| | | | Dirnate* 4 Gm. I.V. | 40.7 | 35.1 | 19.4 | 856 | 1375 | +0.5 |
| | | | Mercuhydrin 2 ml. I.M. | 41.2 | 32.1 | 56.7 | 851 | 1040 | −0.5 |
| M. F. | 64 | Cor pulmonale | Control | 5.7 | 25.0 | 22.0 | 750 | 420 | — |
| | | | Dirnate 8 Gm. P.O. | 74.0 | 74.0 | 26.0 | 660 | 610 | −2.0 |
| | | | Thiomerin 2 ml. I.M.† | 284.0 | 96.8 | 315 | 844 | 2590 | −6.5 |
| R. F. | 57 | Cor pulmonale | Control | 3.0 | 25.0 | 12.0 | 720 | 550 | — |
| | | | Dirnate 8 Gm. P.O. | 4.2 | 55.1 | 21.0 | 1080 | 875 | +2.0 |
| | | | Mercuhydrin 2 ml. I.M. | 13.7 | 64.7 | 60.0 | 796 | 980 | −0.5 |
| H. A. | 57 | ASHD | Control | 105.0 | 72.0 | 132.0 | 1318 | 4120 | — |
| | | | Dirnate 1.04 Gm. I.V. | 103.0 | 58.0 | 148.7 | 1503 | 4240 | +0.5 |
| | | | Mercuhydrin 2 ml. I.M. | 201.0 | 54.0 | 231.0 | 1384 | 5125 | −2.0 |

*When Dirnate was given orally, values for the first or second day of administration are shown, as excretion rates were maximal on one of these two days.

†Preceded by ammonium chloride 6 gm. daily for three days.

*Comparison of Dirnate and Mercurial Diuretics.*—In four patients a comparative study was made of the effect of the oral or intravenous administration of Dirnate and the parenteral injection of mercurial diuretics (Table III). It will be seen that two patients (R. H. and R. F.) in whom sodium excretion was low during control periods did not lose weight after either medication and that the degree of natriuresis was of the same order of magnitude. A rather striking difference in weight reduction and sodium excretion was seen in patient M. F., in whom Thiomerin clearly produced the more marked diuresis; the quantitative contribution of the ammonium chloride "primer" in this instance is not known. The fourth patient (H. A.) doubled his urinary sodium excretion during the twenty-four-hour period following the injection of Mercuhydrin but showed no significant over-all change when given Dirnate intravenously.

*Toxicity.*—Studies in animals[15] have indicated a striking lack of toxicity of Dirnate as determined by histopathologic examination of organs of sacrificed animals, even when large dosages of the drug were administered. Very few side-effects were noticed in our study. Only one patient was unable to tolerate the drug orally because of nausea and vomiting and these symptoms were easily controlled by crushing the tablets and administering them in fruit juice. Several patients complained of tingling of the fingers when the drug was injected intravenously, but no other subjective or objective toxic effects were noted.

## DISCUSSION

While it is apparent from the data presented that Dirnate, in the dosage used, is not a very effective oral diuretic, certain factors must be taken into consideration when its usefulness is gauged. Since all the patients in this study were hospitalized, their very presence in the hospital indicates a degree of salt and water retention not amenable to the usual diuretic measures utilized by the private physician in his office practice. Admittedly, other complications incident to their basic disease state often necessitated their hospitalization; but, in general, the degree of salt and water retention was severe and, more important, many of them were refractory to routine diuretic measures of proven efficacy. This is well demonstrated in the comparative studies of Dirnate and mercurial diuretics in which two patients showed an equally poor response to both agents.

Inhibition of renal carbonic anhydrase disrupts the normal ion-exchange mechanism proposed by Pitts[17] to explain conservation of fixed cation. The role of this mechanism in the acidification of the urine, in bicarbonate reabsorption, and in the excretion of ammonium salts has been recently discussed in detail by Gilman and Brazeau.[18] That the group of carbonic anhydrase inhibitors so far studied has not proved to be very potent is not surprising from a theoretic standpoint. In a comprehensive discussion of carbonic anhydrase inhibitors in 1946, Davenport,[16] on the basis of in vitro studies, stated that, if less than 97 per cent of the enzyme is inhibited, the catalyzed reaction is entirely uninhibited, and that, in order to reduce the rate of catalysis by 90 per cent, more than 99.8 per cent of the enzyme must be inhibited.

It is entirely possible that the arbitrarily selected dosage of Dirnate used in this study was inadequate. The question of dosage, as well as the relationship of level of salt intake to relative magnitude of diuretic response, should be investigated.

Even though it would appear that the carbonic anhydrase inhibitors will not replace mercurial diuretics in the treatment of edema, it is still possible that they will achieve some degree of clinical usefulness. Such a possibility is indicated by Belsky[10] who administered Diamox to thirteen outpatients. Eight patients were enabled to dispense with injections of mercurial diuretics entirely and two other patients exhibited a less frequent need for injections.

## SUMMARY

Dirnate, a carbonic anhydrase inhibitor bearing a close structural relationship to sulfanilamide, was administered to twelve patients exhibiting retention of salt and water incident to cardiac, renal, and hepatic disease.

Although the intravenous administration of the drug induced certain characteristic physiologic responses (increase in urine pH, urine flow, and sodium and potassium excretion), clinical improvement as judged by weight loss was not obtained. Oral administration of Dirnate had no significant diuretic effect.

The ineffectiveness of this agent must be interpreted in the light of the generally severe disease state of the hospitalized patients utilized in this study and their frequent refractoriness to other diuretic measures.

The use of carbonic anhydrase inhibitors in preventing the reaccumulation of edema in ambulant patients and decreasing the necessity for injections of mercurial diuretics should be more extensively investigated.

We wish to express our gratitude to Sharp & Dohme, Inc., West Point, Pa., for the generous supply of Dirnate used in this study and for financial support.

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# EXTREME HYPERFERREMIA IN TWO INSTANCES OF HEMOCHROMATOSIS WITH NOTES ON THE TREATMENT OF ONE PATIENT BY MEANS OF REPEATED VENESECTION

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HEMOCHROMATOSIS is a disease characterized clinically by cirrhosis of the liver, diabetes mellitus, and bronzing of the skin. Pathologically, one finds extensive deposition of hemosiderin throughout the body, especially in the liver, spleen, and pancreas. The clinical and pathologic features have been extensively reviewed by Sheldon in his monograph.[1] The pathogenesis of hemochromatosis is obscure. Since the body lacks a mechanism for the regulatory excretion of iron,[2-6] however, it seems inescapable that increased absorption of iron from the gastrointestinal tract accounts for the large amounts found in the tissues.

The literature contains relatively few reports of serum iron levels in hemochromatosis. In general, values for serum iron in this disease have been at the upper limit of normal or moderately elevated (Table I).

TABLE I. SERUM IRON VALUES IN HEMOCHROMATOSIS

| AUTHORS | CASE NO. | SERUM IRON (μG %) |
|---|---|---|
| Heilmeyer and Plötner[7] | 1 | 199 |
| Moore et al.[8] | 1 | 101 |
| Rath and Finch[9] | 1 | 233 |
| | 2 | 170 |
| | 3 | 235 |
| | 4 | 245 |
| | 5 | 250 |
| | 6 | 250 |
| | 7 | 204 |
| | 8 | 204 |
| | 9 | 220 |
| Lemaire et al.[10] | 1 | 150 |
| | 2 | 135 |
| Cartwright[11] | 1 | 147 |

Some of the patients described by Rath and Finch[9] exhibited extreme degrees of hyperferremia in the terminal phase of the disease. This was interpreted as representing a terminal necrobiosis and consequent release of iron.

Two instances of hemochromatosis are reported. Both patients showed extreme degrees of hyperferremia, and in one of them, this was not a terminal event. In addition the latter patient was, and is still being subjected to repeated phlebotomy as a therapeutic measure.

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Received for publication, Dec. 8, 1953.

Material for this paper was taken in part from a thesis which was submitted to the Graduate Faculty of the University of Minnesota in partial fulfillment of the degree of Ph.D. in Medicine which was granted in June, 1952.

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## METHODS

In Case 1, the serum iron was measured by a modification of the method of Moore and associates.[12] This method is one in which ferric iron reacts with thiocyanate to produce a red color which is determined colorimetrically.

In Case 2, serum iron determinations were done according to the modification of the method of Barkan and Walker[13] in which ferrous iron reacts with orthophenanthrolene also to produce a red color which is determined colorimetrically. Due to its greater ease of performance the latter method is now standard in this laboratory. In twenty-two normal women, values for serum iron ranged from 59 to 190 $\mu$g per 100 ml. of serum with a mean value of 115.1. In thirty-four normal men, the range was 65 to 218 $\mu$g per 100 ml. with a mean value of 121.5. The difference between values for men and women was not significant statistically. Mean value for the entire group of fifty-six normal persons was 119.1 $\mu$g per 100 ml. with an S.D. of 38.0. To test the reliability of the method, duplicate determinations were carried out in 146 consecutive pairs of serum samples. The standard deviation of the determination was found to be 5.74 $\mu$g per 100 ml.

## REPORT OF CASES

CASE 1.—Ke., U. H. No. 734940, a 62-year-old white male, first noted the onset of weakness, tiredness, and loss of weight in 1942. Early in 1943, he noted a mass in the right upper quadrant of his abdomen which increased progressively in size; he had lost approximately 30 pounds in weight. A brother of the patient had died elsewhere in 1941; autopsy had revealed generalized peritonitis and hemochromatosis.

TABLE II. LABORATORY STUDIES, PATIENT KE., CASE NO. 1

| | SEPT., 1943 | DEC., 1944 | FEB., 1946 |
|---|---|---|---|
| Urine: | | | |
| Glucose | 4+ | Neg. | Tr to 2+ |
| Acetone | Neg. | Neg. | Neg. |
| Blood: | | | |
| Hbg. in gm. per 100 ml. | 14.4 | 14.0 | 12.8 |
| WBC per c. mm. | 12,800 | 5,280 | 10,800 |
| Glucose in mg. per 100 ml. | 318 | 83 and 160 | 139 |
| $CO_2$ combining power in vol. % | | | 60 |
| Chlorides in mg. per 100 ml. | | | 627 |
| Urea nitrogen in mg. per 100 ml. | | | 13 |
| Liver Function: | | | |
| Serum bilirubin in mg. per 100 ml. | | | |
| 1 min. | 0.04 | 0.1 | |
| Total | 0.5 | 0.9 | 0.85 |
| Bromsulfthalein, % retention 45 min. after 5 mg. per Kilogram body weight | 7 | | 14 |
| Cephalin-cholesterol flocculation, 24 hours | 0 | 0 | 2+ |
| Thymol turbidity, units | | | 4 |
| Cholesterol: Total in mg. per 100 ml. | 215 | 166 | 168 |
| Esters % | 75 | | 61 |
| Proteins in Gm. per 100 ml. | | | |
| Total | 6.7 | 6.5 | |
| Albumin | 3.4 | 3.7 | |
| Globulin | 3.0 | 2.9 | |
| Urine urobilinogen in mg. per 24 hours | 3 to 7 | 5.7 | 35 |

Physical examination at the time of his first hospital admission in September, 1943, revealed a well-developed white male, chronically ill and obviously malnourished. The blood pressure was 115/75. There was increased pigmentation of the hands and face. The heart was slightly enlarged to the left, but was otherwise normal. The liver was palpable 6 cm. below the costal margin and 9 cm. below the xiphoid process. Its surface was firm, but not nodular or tender. The remainder of the physical examination was within normal limits.

Table II contains the essential laboratory data for this and the two subsequent admissions. A glucose tolerance test, performed on the first admission, was characteristic of diabetes mellitus. A gastrointestinal x-ray series and a barium enema revealed no abnormalities. A skin biopsy was negative for iron. A liver biopsy revealed an increase in connective tissue and a large amount of brown pigmentation which gave a positive histochemical stain for iron.

The diabetic state was easily controlled on a diabetic diet and approximately 30 units of insulin per day. He was discharged in an improved condition approximately one month after admission.

He was again admitted to the hospital briefly in December, 1944 for re-evaluation of his status which was found to be essentially unchanged. Thereafter, he was seen in the outpatient clinic at intervals of two to three months. His general condition remained satisfactory.

On Feb. 15, 1946, he was admitted to the hospital in a comatose state. About one week earlier he had developed weakness and anorexia. Despite the loss of appetite, he continued to take the usual doses of insulin. He gradually became disoriented, incontinent, and finally comatose. He was first given glucose intravenously by his private physician and subsequently insulin was administered. Neither effected any change in his condition.

On admission the patient was comatose and decidedly more emaciated than he had been on previous admissions. Table II shows the results of the routine laboratory examinations on this admission. There was no laboratory evidence of acidosis, hypoglycemia, azotemia, or cholemia. Roentgenograms of the chest and of the skull were not remarkable. An electrocardiogram showed a sinus tachycardia with low voltage of the QRS complex in the limb leads. The sedimentation rate was 74 mm. in one hour. The venous pressure was equivalent to 7 cm. of water.

During the hospital stay, the patient remained afebrile, the chest remained clear, and no evidence of acidosis, hypoglycemia, or uremia developed. However, he became increasingly stuporous.

Neurological examination revealed no nuchal rigidity. The left biceps jerk was greater than the right, and the right knee jerk greater than the left. There was a positive Gordon test on the right. A lumbar puncture was done and showed normal pressure. The fluid was clear and contained no cells. The patient was given supportive therapy and insulin in dosages required to maintain the blood sugar at, or somewhat below, 200 mg. per 100 ml. Nevertheless, he remained comatose and died on February 18, four days after admission.

Of special interest was the value for serum iron which was measured on the day preceding death. This value was found to be 4,020 $\mu$g or 4.02 mg. per 100 ml. of serum. Unfortunately the determination could not be repeated, as the patient had died by the time the extremely high value became known. Two control sera, however, measured simultaneously, gave normal results.

*Autopsy Findings.*—Necropsy was performed by Dr. John Coe, of the Department of Pathology, to whom we are indebted for the following report. There was generalized bronzing of the skin and a slight scleral icterus. The heart weighed 350 grams. The coronary arteries were moderately sclerotic but patent throughout. The lungs and spleen were normal. The liver weighed 1,885 grams. Its external surface was finely nodular. The right lobe was very firm, the left lobe somewhat softer and coarser. The caudate lobe contained a hard, white, infiltrating mass, approximately 2 by 4 by 3 cm., which did not extend into the portal structures. The gall bladder appeared large, as though it had been previously distended. No stones were found. The common bile duct appeared normal, but the hepatic duct proximal to the junction with the cystic duct was grossly thickened and partially obstructed. The portal vein and the hepatic artery were not occluded.

The pancreas was replaced almost entirely by fat. The few small remaining areas of pancreatic tissue were dark brown in color. The adrenals and kidneys appeared normal. The periaortic, mesenteric, and portal lymph nodes were enlarged and dark brown, but grossly free of metastatic tumor.

*Microscopic.*—Sections of the right and left lobe of the liver revealed severe portal cirrhosis with some new bile duct proliferation. The liver architecture was completely disarranged. In all areas, the cells contained large amounts of pigment which gave a positive test for iron. The mass in the caudate lobe consisted primarily of connective tissue containing well developed, erratically arranged bile ducts, characteristic of cholangioma.

The thickening of the hepatic duct proved to be an adenocarcinoma which had invaded the cystic duct and surrounded the hepatic artery and vein, although not invading them. Several small arteries had been invaded.

The pancreas was extensively infiltrated with fat. Very few acini were seen and those present contained large amounts of iron pigment. Only one islet could be identified. The kidneys were normal except for iron pigment in the epithelial cells of the collecting tubules. The lungs showed a few patchy areas of atelectasis and bronchopneumonia. The thyroid acinar cells contained large amounts of iron pigment. The lymph nodes were hyperplastic and contained many giant cells which were also filled with iron pigment. There was no suggestion of metastatic carcinoma.

Iron containing pigment was also present in the spleen, the adrenal cortex, and the myocardium.

The brain was examined by Dr. A. B. Baker and Dr. H. H. Noran, of the Division of Neuropathology. There was a small amount of subarachnoid bleeding over the tip of the right frontal lobe. The results of gross examination were otherwise normal.

Microscopically there were numerous small focal areas of perivascular demyelinization, scattered diffusely through the white matter primarily, with a small number scattered through the gray matter. It was thought that this did not represent the usual picture of hemochromatotic pigmentation of the nervous system.

*Summary.*—A patient with proved hemochromatosis presented the terminal picture of progressively deepening coma without evidence of diabetic acidosis, uremia, or adrenal insufficiency; nor were the usual features of hepatic coma present. An extremely high serum iron was found shortly before death. Necropsy revealed, in addition to hemochromatosis and cirrhosis, a cholangioma and a carcinoma of the hepatic duct. No distant metastases were found.

CASE 2.—Ur., U. H. No. 783024, a 43-year-old physician, was first seen on Aug. 22, 1947, with complaints of diarrhea and epigastric fullness. About two weeks previously, he had noted anorexia, vomiting, diarrhea, and dark urine. A short time later scleral icterus appeared. All symptoms and signs disappeared within a week. Shortly thereafter the patient discovered that his liver was enlarged. He had been exposed to many patients with infectious hepatitis in his community during the preceding three months. He had had mild hypertension since 1931. In 1937 he had experienced an episode originally thought to be a coronary occlusion, but later believed to have been caused by intercostal neuritis.

The patient was a slightly obese white man. Blood pressure was 148/86. A diffuse brown pigmentation of the skin was described as "bronze" by some examiners; it was more intense in exposed areas. There was a malar flush, and there were numerous "spider nevi" over the thorax and upper arms. The lungs were clear, and a moderate left ventricular type of cardiac enlargement was demonstrated. The liver edge was palpable 11 cm. below the right costal margin in the mid-clavicular line. Its surface was smooth and nontender. The tip of the spleen was palpable on deep inspiration. No other abnormal findings were noted.

Urinalysis was negative. The hemoglobin was 14.2 Gm. per 100 ml. of blood and the leukocyte count and differential were normal. The sedimentation velocity was 47 mm. in one hour. (Westergren method.) Serologic tests for syphilis were negative. Fasting blood sugar was 94 mg. per 100 ml. of blood. A glucose tolerance test was done. Blood sugar rose to 300 mg. per 100 ml. at 90 minutes, returned to 144 mg. per 100 ml. at 150 minutes. This was thought to be compatible with a latent diabetes mellitus. Results of liver function studies are given in Table IV.

TABLE III. IRON METABOLISM STUDIES, PATIENT UR., CASE 2

| DATE | 9-3-47 | 10-4-47 | 12-9-47 | 1-23-48 | 3-19-48 | 9-15-48 | 12-7-48 | 2-17-49 | 2-4-50 | 12-13-50 | 6-8-51 | 7-31-51 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Hemoglobin in Gm./100 ml. | 14.9 | 12.5 | 15.5 | 13.7 | 13.3 | 13.0 | 14.0 | 12.25 | 12.3 | 13.6 | 17.5 | 14.0 |
| RBC in millions per c. mm. | 4.57 | 3.52 | 3.88 | 3.62 | 3.36 | 3.65 | 4.45 | 4.02 | 5.16 | 5.02 | 5.16 | |
| Reticulocytes (%) | 0.5 | 4.0 | 2.0 | 3.6 | 4.4 | 4.3 | 2.2 | 3.0 | 2.1 | 2.1 | | 4.80 |
| Serum iron in $\mu$g per 100 ml. | 420 | 362 | | *8,000* | | 44 | | | | | 144 | |
| Blood removed, cumulative total in liters | 1.05 | 4.30 | 9.43 | 14.93 | 21.90 | 47.20 | 51.60 | 59.60 | 77.2 | 89.4 | 94.0 | 96.4 |
| Iron removed, cumulative total in Gm. | 0.52 | 1.86 | 4.32 | 6.85 | 10.86 | 21.83 | 23.80 | 27.30 | 36.02 | 41.11 | 43.51 | 44.79 |

The electrocardiogram was normal. Chest roentgenogram revealed moderate enlargement of the left ventricle, but the lung fields were clear. Barium swallow disclosed no evidence of esophageal varices.

Liver biopsy was carried out at this time and was interpreted by Dr. W. W. Walker of the Department of Pathology as showing an advanced cirrhosis. The most striking feature was the presence of large amounts of coarse brownish pigment chiefly within the portal areas, but also present in considerable amounts within the liver cells. This pigment demonstrated the staining characteristics of hemosiderin and the diagnosis of hemochromatosis was considered established.

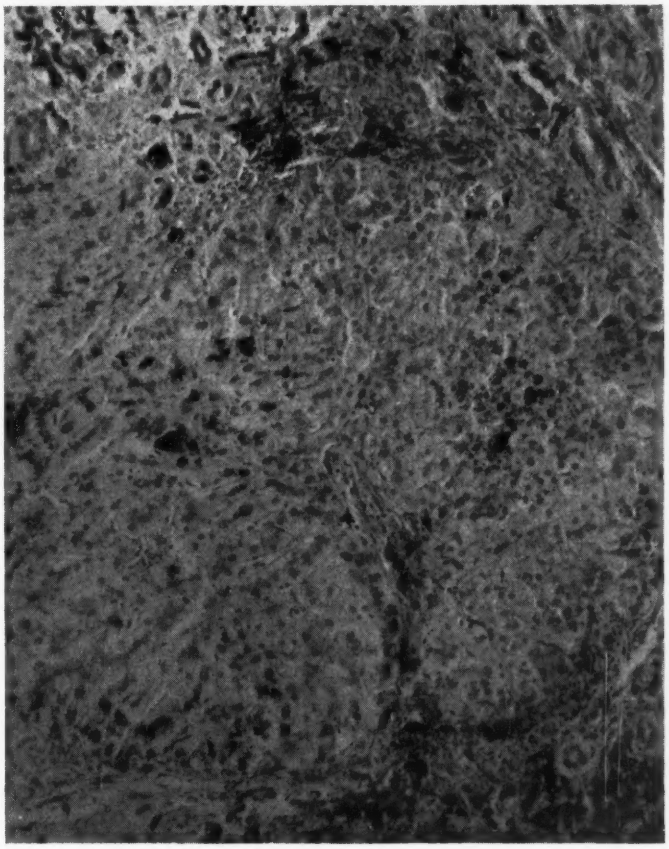

Fig. 1.—Liver biopsy, Patient Ur., August, 1947. Before therapy. Prussian blue stain. (×100.)

*Therapy.*—In an attempt to remove at least some of the stored iron, treatment by means of repeated bleeding was decided upon. This had been suggested by Dr. William Davis,[14, 15] and by Dr. Paul Hahn,[16] both of whom had attempted it previously in patients with hemochromatosis. Accordingly, blood-letting was begun on Sept. 3, 1947, with the removal of 1,050 ml. of blood. Three days later another 450 ml. was removed. These procedures were well tolerated. The hemoglobin, 14.9 Gm. per 100 ml. of blood initially, had decreased to 11.0 Gm. by September 9. The erythrocyte count had similarly decreased from 4.57 million per cubic millimeter to 3.15 million over a similar period.

Phlebotomy has been repeatedly carried out up to the time of this report. A low iron diet was also advised but the patient followed this only perfunctorily.

Data showing the effects of blood-letting on hemoglobin, erythrocyte count, reticulocyte percentage, and serum iron are presented in Table III. These observations were made between Sept. 3, 1947 and July 31, 1951, a period of forty-seven months. During this period, a total of 96,400 ml. of blood, with a calculated total iron content of slightly less than 45 Gm., was removed from the patient's circulation.

Despite the removal of such a large amount of blood and some decrease in iron intake, the patient's hemoglobin remained at a level of approximately 13 Gm. per 100 ml. of blood during the entire period of observation. The lowest value obtained was 11.0 Gm., and that was noted on only one occasion. A bone marrow aspiration was performed on Dec. 9, 1947, approximately three months after institution of phlebotomy. Normoblastic hyperplasia was evident. Pigment-containing macrophages were found in increased numbers.

The patient showed definite subjective improvement on this regime. He was last examined by the authors on June 8, 1951. At that time, he reported a definitely increased feeling of well-being and more energy, allowing him to maintain an active surgical practice. He was also free of the diarrhea and the feeling of epigastric fullness, of which he had complained when first seen. Cutaneous pigmentation had entirely disappeared. The liver was now palpable only 6 cm. below the costal margin in the midclavicular line, a definite decrease in comparison with the findings on initial examination. There were only four small spider nevi in the left deltoid region.

Due to difficulties with his arm veins, he had found it necessary to interrupt the phlebotomies temporarily from time to time. At the time of the last examination, no blood had been withdrawn for about two months. It is of interest to note that his hemoglobin had risen to 17.5 Gm. during this two-month interval.

The results of liver function studies carried out prior to onset of bleeding and during the course of treatment are shown in Table IV. It should be pointed out that while in 1947 the serum protein fractionation was done by the Howe method, which measures alpha-globulin as albumin, in 1950 the Majoor technique, which measures alpha-globulin as globulin, was used.

TABLE IV. LIVER FUNCTION STUDIES, PATIENT UR., CASE 2

| | AUG. 1947 | DEC. 1950 | JUNE 1951 |
|---|---|---|---|
| Serum bilirubin—1 min. in mg. per 100 ml. | 0.3 | 0.3 | 0.3 |
| Total | 0.6 | 0.7 | 0.7 |
| Thymol turbidity in units | 6.6 | 7 | 6 |
| Cephalin-cholesterol flocculation (24 hours) | 2+ | 3+ | 2+ |
| BSP retention in % (5 mg. per kg.) | 12 (45 min.) | 13 (60 min.) | 12 (45 min.) |
| Total cholesterol in mg. per 100 ml. | 170 | 213 | |
| Ester cholesterol in % | 65 | 83 | |
| Total serum protein Gm. per 100 ml. | 9 | 8.8 | 8.3 |
| Albumin Gm. per 100 ml. | 3 | 3.2 | |
| Globulin Gm. per 100 ml. | 6 | 5.6 | |

Despite unquestionable clinical improvement, there had been little or no significant change in measurable hepatic function. The serum albumin, however, showed a slight increase (when considered in the light of the two different techniques employed) during the period of treatment, despite the very large amounts of protein withdrawn by the many bleedings.

*Iron Studies* (Table III).—Before the institution of therapy, the patient's serum iron was 420 μg per 100 ml. of serum. Seven observations during the succeeding month ranged from 360 to 415 μg per 100 ml. No further serum iron determinations were made until Jan. 23, 1948, about four and one-half months after blood-letting was begun. Five

samples were taken on this and the following day in conjunction with a radioactive iron absorption test to be discussed later. The iron content of these sera varied between 7,040 and 8,000 μg per 100 ml. Each sample was analyzed at least twice and two of them three times. Similar results were obtained in every instance; therefore contamination seems an unlikely explanation of the hyperferremia. After approximately one year of blood-letting, the patient's serum iron had decreased to 44 μg per 100 ml., while the last determination made gave an essentially normal value, 144 μg per 100 ml.

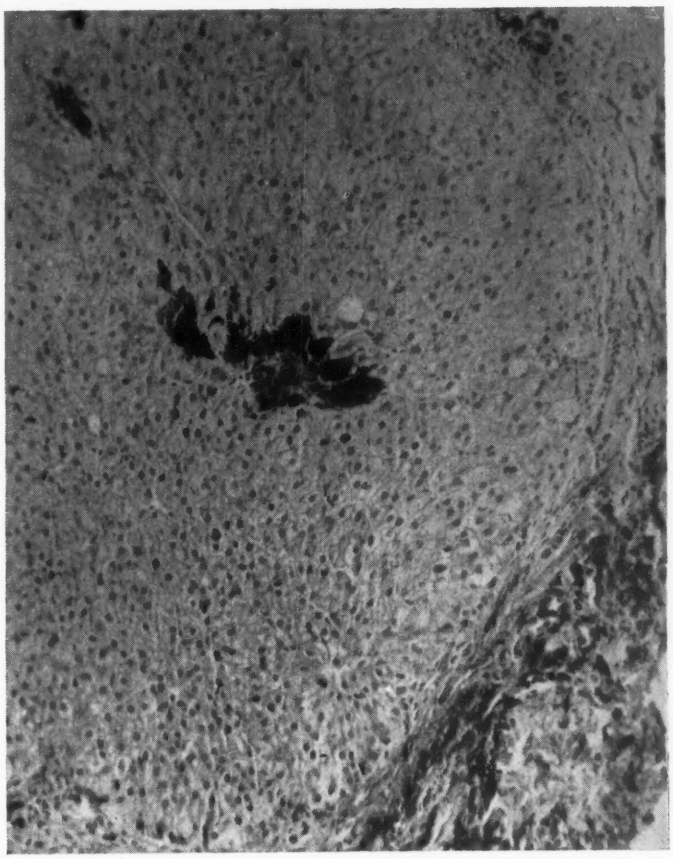

Fig. 2.—Liver biopsy, Patient Ur., September, 1948. Thirteen months after beginning of blood-letting. At this time more than 47 L. of blood, containing more than 21 Gm. of iron had been removed. Prussian blue stain. (×100.)

Liver biopsy has been performed on two occasions in this patient. The first specimen was obtained in August, 1947, when the patient was first seen. The second was obtained in September, 1948, after treatment had been in progress for approximately one year. Slightly more than 21 Gm. of iron had been removed by phlebotomy at the time of the second biopsy. Both specimens gave a strong positive histologic stain for iron, but some observers believed that this was definitely less intense in the second one (Figs. 1 and 2). Recently, an attempt was made to measure quantitatively the iron content of these two biopsy specimens. The only tissues available were those imbedded in paraffin blocks, from which the original microscopic sections had been cut. The paraffin was dissolved with xylene which was allowed to evaporate, leaving behind the small, dehydrated, fat-free pieces of tissue. These were weighed on a Kuhlmann precision balance which has a

sensitivity of 0.01 mg. A modification of Schaefer's method[17] was used for the determination of the iron in these tissues. The same procedures were applied to nine histologically normal livers. The results of these determinations are given in Table V.

The amounts of tissue available were exceedingly small; the absolute amounts of iron to be dealt with were consequently minute. The colorimetric determination of iron becomes progressively less accurate as the amount of iron decreases, and any error introduced by contamination is magnified. Furthermore, the small amounts of tissue precluded the determination of hemoglobin and therefore of hemoglobin iron. For these reasons, it is not believed that these data are particularly accurate. It is believed, however, that they demonstrate adequately the remarkable iron storage in the liver of our hemochromatotic patient. The significance of the 12 per cent decrease in liver iron in the patient after one year of blood-letting is doubtful in view of the limitations of the technique.

TABLE V. RESULTS OF IRON DETERMINATIONS ON LIVER BIOPSY SPECIMENS (SEE TEXT)

| LIVER BIOPSY | WEIGHT OF DRIED SPECIMEN IN MG. | IRON CONTENT IN MG. PER 100 GM. OF DRY LIVER |
|---|---|---|
| *Specimen* | | |
| Patient Ur. (Aug., 1947) | 4.53 | 2,428.0 |
| Patient Ur. (Sept., 1948) | 4.11 | 2,114.0 |
| Normal liver | 6.64 | 28.6 |
| Normal liver | 5.93 | 40.5 |
| Normal liver | 4.77 | 56.6 |
| Normal liver | 1.18 | 110.0 |
| Normal liver | 1.17 | 25.6 |
| Normal liver | 2.96 | 6.76 |
| Normal liver | 4.61 | 13.0 |
| Normal liver | 4.58 | 28.4 |
| Normal liver | 1.75 | 120.0 |
| Average normal liver | | 37.7 |

*Summary.*—A 43-year-old physician with hemochromatosis has been treated by means of repeated bleeding for a period of more than six years. During the first four years, a total of 96,400 ml. of blood, containing approximately 45 Gm. of iron, was removed. Four and one-half months after institution of this therapy, the serum iron level was found to be remarkably high, falling to a rather low level at the end of one year. Symptomatic improvement and decrease in cutaneous pigmentation were noted. The liver also decreased in size.

## DISCUSSION

In the first patient a terminal hyperferremia of extreme degree occurred. This phenomenon has been described by Rath and Finch,[9] who believed that a release of iron had occurred from liver and other tissues undergoing necrobiosis as a terminal event. This may well have been the case in this patient.

The second patient, however, presents features not previously described. Of particular interest is the fact that over a period of almost four years, more than 96 L. of blood, containing approximately 45 Gm. of iron, were removed from this patient. Despite this, his hemoglobin remained at a relatively constant level, thus attesting to the availability of the abnormally large iron stores in this disease. The extreme hyperferremia (7,000 to 8,000 $\mu$g per 100 ml.) noted four and one-half months after bleeding was begun is likewise of great interest. The explanation for the phenomenon can only be conjectural. The authors favor the possibility that the repeated bleeding had mobilized such a large amount of iron

from the body stores that hemoglobin regeneration, even though rapid, was unable to keep up with the rate of mobilization of iron and that it therefore "overflowed" into the blood serum. In contrast to the first patient, and to the patients of Rath and Finch, it should be emphasized that in this instance at the time this high value was obtained, the patient felt very well and was experiencing no untoward symptoms of any kind. It has been suggested that during intravenous iron therapy, toxic symptoms occur when the saturation limit of the specific iron-binding serum protein is exceeded[18-20] That limit was undoubtedly greatly exceeded in this patient, and yet no toxic symptoms occurred. After about one year of repeated bleeding, the serum iron had decreased to 44 $\mu$g per 100 ml., a value generally associated with a moderately severe iron deficiency state. These findings suggest that mobilization of the stored iron took place initially at a relatively rapid rate, but that as the total amount of iron removed from the body increased, the ease with which stored iron was mobilized decreased progressively.

At the time of the low serum iron value, presumably mobilization of iron was lagging behind hemoglobin regeneration, resulting in a relative deficiency in the serum iron value. More recently, the serum iron was found to be normal. This value, obtained after a two month period during which no phlebotomy was done, testifies to the remarkable availability of the iron stores in this patient. There is evidence, however, which indicates that the most recently stored iron is most readily available and that iron stored in the more distant past is less easily mobilized.[21, 22] The extent to which abnormally stored iron can be removed from our patient remains to be determined.

A small dose of radioactive iron was given orally to the patient in Case 2. After an interval of thirty-five days, the peripheral blood contained 16.6 per cent of the administered dose. Under similar conditions, Balfour and co-workers[16] found only 1.8 per cent of the administered dose in the peripheral blood of a normal individual. Interestingly, their patient with hemochromatosis showed a value of 0.8 per cent even after the stimulus of repeated bleeding. Although these authors referred to this procedure as a measurement of iron *absorption,* it is actually only a method of determining iron *utilization,* for no account is taken of iron which may have been absorbed, but not incorporated into hemoglobin. Attempting to measure iron absorption accurately, Dubach and her associates[23] fed normal subjects test doses of radioactive iron in an amount of 1 mg. of iron per kilogram of body weight. In each subject, the amount of iron utilized in the formation of hemoglobin was calculated and in addition, a very careful collection of stools was carried out until the twenty-four-hour specimen contained less than 0.5 per cent of the administered dose. This usually required six or seven days, but in two instances as much as twelve days. Thus:

Administered iron – iron recovered in feces = absorbed iron, and
Administered iron – (iron recovered in feces + iron utilized in hemoglobin formation) = iron absorbed, but stored

In ten determinations in eight subjects, they found values for iron absorption ranging between 2 and 21 per cent of the administered dosage. In all but one of the determinations, there was evidence that a measurable quantity of

iron was absorbed, but not utilized. This amounted to 17 per cent of the administered dosage in one instance and was over 10 per cent in three others. Their patient with hemochromatosis, studied by the same methods, absorbed 21.5 per cent of the administered dosage and utilized only 2 per cent for hemoglobin formation. In other words, 19.5 per cent was absorbed and added to iron stores. Our patient, on the other hand, *utilized* 16.6 per cent of the administered dosage from hemoglobin formation. We can thus state that *at least* 16.6 per cent of the iron was absorbed. It is true that this study was made four and one-half months after blood-letting was initiated and that utilization of absorbed iron might thus be expected to be more complete because of stimulation of hematopoiesis. There was, however, no hematologic evidence to suggest iron deficiency at that time and the remarkable hyperferremia, previously alluded to, was present both before and after administration of the radioactive iron. It seems likely that absorption of an amount of iron significantly in excess of that utilized took place and that iron retention exceeded that expected in a normal individual. This serves to strengthen the concept[24] that the gastrointestinal tract is of primary importance in the genesis of hemochromatosis.

## SUMMARY

Two patients with hemochromatosis were studied. Both presented remarkable degrees of hyperferremia. In one instance this was a terminal event, but in the second patient it occurred while the patient was free of untoward symptoms.

In the second patient repeated venesections were performed in an attempt to remove at least a portion of his greatly increased iron stores. During the first four years of treatment, more than 96 L. of blood, containing almost 45 Gm. of iron, were removed. Concomitantly, improvement in general status, decrease of pigmentation, and decrease in liver size were noted. Liver function tests remained essentially unchanged.

Radioactive iron studies in the second patient suggested that he absorbed a greater percentage of an orally administered dose of iron than do most normal individuals. This is in keeping with current concepts of the pathogenesis of hemochromatosis.

The authors wish to express their thanks to Dr. C. J. Watson for the opportunity to study the second patient and for many valuable suggestions with regard to the study and the preparation of the manuscript.

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# THE RELATIVE INCIDENCE OF ANTI-HEMOPHILIC GLOBULIN (AHG), PLASMA THROMBOPLASTIN COMPONENT (PTC), AND PLASMA THROMBOPLASTIN ANTECEDENT (PTA) DEFICIENCY: A STUDY OF FIFTY-FIVE CASES

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UNTIL recently, hemophilia was generally considered a single disease entity with a coagulation defect characterized by a prolonged clotting time and decreased prothrombin consumption. The description of hemophilia-like conditions from which blood or plasma corrected the coagulation defect in hemophilia made this assumption untenable.[1-8] Besides the classical type of hemophilia caused by a deficiency of Anti-Hemophilic Globulin (AHG), there is adequate evidence for the existence of at least two additional conditions with clinical and laboratory features similar to hemophilia. The first disease entity was called Plasma Thromboplastin Component (PTC) deficiency by White and associates,[3] who thoroughly investigated a patient with this condition and succeeded in partially separating PTC from other normal plasma components. PTC deficient plasma contains normal quantities of AHG. The second entity was described by Rosenthal and colleagues[5] as Plasma Thromboplastin Antecedent (PTA) deficiency. PTA deficient plasma corrected the clotting defect in AHG and PTC deficiency.

The present study was undertaken to determine the relative incidence of AHG, PTC, and PTA deficiency.

## MATERIALS AND METHODS

A total of fifty-five patients previously diagnosed simply as hemophilia were re-studied with the following tests:

*Coagulation Time* (modified Lee White Method).—One milliliter of blood was placed in each of two glass tubes (75 by 8 mm.). Starting ten minutes after venipuncture, the first tube was tilted every minute until clotted. The second tube was started when the end point of the first one was reached. The end point of the second tube was recorded as clotting time of venous blood. Normal range: twelve to twenty minutes.

*Bleeding Time.*—Method of Ivy.[9]

*Prothrombin Time.*—Method of Quick.[10]

*Prothrombin Concentration.*—Previously described modification of the two-stage method of Ware and Seegers.[11] During the first stage of this test, prothrombin was converted to thrombin with an incubation mixture containing thromboplastin and calcium. In the second stage, the maximum thrombin yield was determined by measuring the time needed to clot a standard fibrinogen solution. This time is an index of thrombin concentration which is computed in units per milliliter from a standard table. By definition, a unit of thrombin is derived from a unit of prothrombin. The concentration of prothrombin in normal human plasma is 250 to 350 units per milliliter. For details of the method, the reader is referred to the pertinent literature.

Received for publication, Dec. 14, 1953.

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*Capillary Fragility.*—Rumpel-Leede test.[12]

*Platelet Count.*—Method of Rees-Ecker.[13]

*Clot Retraction.*

*Prothrombin Consumption. One-Stage Method.*—Two milliliters of blood were collected in a 75 by 8 mm. dry glass tube and placed in a water bath at 37° C. for three hours. One-tenth milliliter of the supernatant serum was then added to a mixture of 0.1 ml. of normal $BaSO_4$ treated plasma (used as fibrinogen substrate), 0.1 ml. of thromboplastin solution and 0.1 ml. 1/40 M $CaCl_2$ solution. The time interval between serum addition and appearance of a clot is the serum prothrombin time. In normal blood this value is above 23.0 seconds. Serum prothrombin times below 20.0 seconds indicate decreased prothrombin consumption.

*Prothrombin Consumption. Two-Stage Method*[14].—Blood collected in a siliconized syringe was distributed into a series of 75 by 8 mm. dry glass tubes calibrated to 2.0 ml. and placed in a water bath at 37° C. At fifteen-minute intervals 0.2 ml. of 3.8 per cent sodium citrate solution was added to successive tubes and the citrated serum obtained by centrifugation. The concentration of prothrombin was then determined on each serum sample with the modified two-stage method briefly outlined above.

TABLE I. OCCURRENCE OF AHG, PTC, AND PTA IN NORMAL $BaSO_4$ TREATED PLASMA AND NORMAL SERUM

| | AHG | PTC | PTA |
|---|---|---|---|
| Normal $BaSO_4$ plasma | Present | Absent | Present |
| Normal forty-eight-hour serum | Absent | Present | Present |

Normal $BaSO_4$ plasma was prepared by adding 100 mg. of $BaSO_4$ (Merck & Co.) to each milliliter of normal oxalated plasma. This mixture was gently shaken at room temperature for five minutes and the $BaSO_4$ centrifuged out at 2,000 r.p.m. for ten minutes.

Normal forty-eight-hour-old serum was obtained from normal blood collected under sterile conditions and allowed to clot spontaneously in glass tubes at 37° C. for forty-eight hours.

TABLE II. EFFECT OF NORMAL $BaSO_4$ TREATED PLASMA AND NORMAL FORTY-EIGHT-HOUR-OLD SERUM ON THE CLOTTING DEFECT OF AHG, PTC, AND PTA DEFICIENCY

| | NORMAL $BaSO_4$ PLASMA | NORMAL 48-HOUR SERUM |
|---|---|---|
| AHG deficiency | Corrective | Ineffective |
| PTC deficiency | Ineffective | Corrective |
| PTA deficiency | Corrective | Corrective |

*Differential Diagnostic Tests.*—Since AHG, PTC, and PTA deficiency cannot be separated from each other with the tests listed above, additional differentiating methods had to be devised. The physicochemical properties of AHG, PTC, and PTA and the behavior of the three factors during spontaneous coagulation of normal blood have been applied to these methods (Table I).

AHG and PTA are not absorbed by $BaSO_4$, while PTC can be completely removed from normal oxalated plasma with this salt. Normal serum contains PTC and PTA but is devoid of AHG activity. Hence the clotting defect in AHG deficiency can be restored to normal with normal $BaSO_4$ treated plasma but not with normal serum; PTC deficiency is unaffected by normal $BaSO_4$ treated plasma but responds to normal serum; PTA deficiency is corrected by normal $BaSO_4$ treated plasma and by normal serum (Table II).

The diagnosis of the single cases was based upon the ability or failure of normal $BaSO_4$ treated plasma and normal 48-hour-old serum to correct the abnormal clotting time and prothrombin consumption in each case. The use of 48-hour-old normal serum is preferable to fresh serum because it is free of thrombin and AHG and contains only traces of labile factor and prothrombin. One-tenth milliliter of normal plasma, normal serum, normal $BaSO_4$ treated plasma, and 0.9 per cent NaCl solution were placed in the test tubes to be used for determination of the coagulation time. Two-tenths milliliter of the same

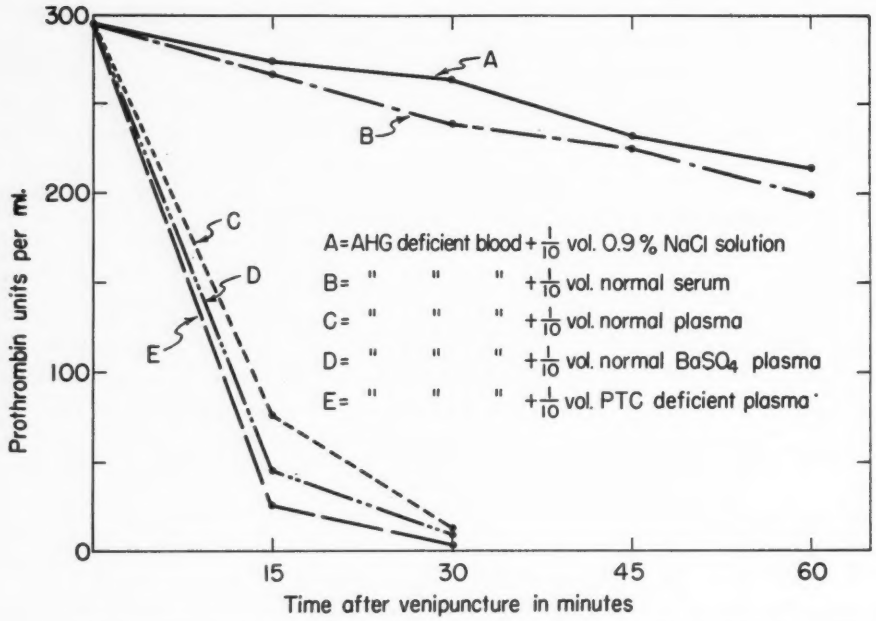


Fig. 1.—Two-stage prothrombin consumption studies in a case of AHG deficiency.

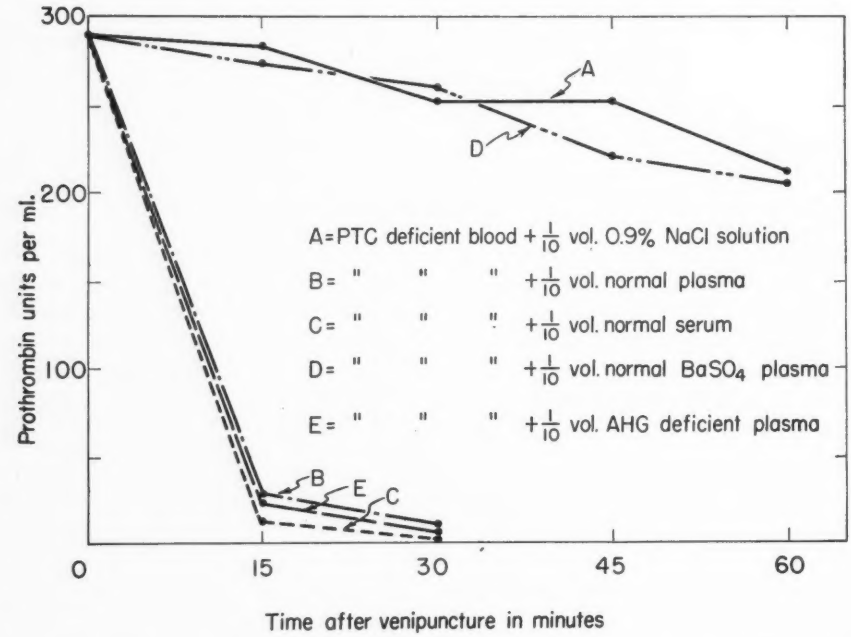


Fig. 2.—Two-stage prothrombin consumption studies in a case of PTC deficiency

reagents was placed in the test tubes to be used for prothrombin consumption. The patient's blood was collected in siliconized syringes through Arquad 2-C coated needles and distributed into the tubes in aliquots of 1.0 ml. and 2.0 ml. for determination of clotting time and prothrombin consumption, respectively. All tubes were immediately inverted twice to ensure adequate mixing. Coagulation times and prothrombin consumption tests were then carried out with the methods described previously.

## RESULTS

The only consistent abnormalities were a prolongation of the clotting time and a diminished consumption of prothrombin during spontaneous coagulation in glass tubes. All other tests were within normal range except for an occasional prolongation of the bleeding time in infants; this is a well-known observation and is believed to be on the basis of injury of vessels of larger caliber than the capillaries.

The results obtained with the differential diagnostic tests in a case of AHG deficiency are presented in Table III and Fig. 1.

TABLE III. COAGULATION TIME AND ONE-STAGE PROTHROMBIN CONSUMPTION TEST IN A CASE OF AHG DEFICIENCY

| | | | | | | |
|---|---|---|---|---|---|---|
| AHG deficient blood | (ml.) | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Nine-tenths per cent NaCl solution | (ml.) | 0.1 | | | | |
| Normal plasma | (ml.) | | 0.1 | | | |
| Normal $BaSO_4$ plasma | (ml.) | | | 0.1 | | |
| Normal 48-hour serum | (ml.) | | | | 0.1 | |
| PTC deficient plasma | (ml.) | | | | | 0.1 |
| Clotting time (in minutes) | | 90 | 15 | 16 | 76 | 18 |
| Serum prothrombin time 3 hours after venipuncture (in seconds) | | 14.8 | 25.0 | 27.2 | 14.0 | 25.9 |

TABLE IV. COAGULATION TIME AND ONE-STAGE PROTHROMBIN CONSUMPTION TEST IN A CASE OF PTC DEFICIENCY

| | | | | | | |
|---|---|---|---|---|---|---|
| PTC deficient blood | (ml.) | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Nine-tenths per cent NaCl solution | (ml.) | 0.1 | | | | |
| Normal plasma | (ml.) | | 0.1 | | | |
| Normal $BaSO_4$ plasma | (ml.) | | | 0.1 | | |
| Normal 48-hour serum | (ml.) | | | | 0.1 | |
| AHG deficient plasma | (ml.) | | | | | 0.1 |
| Clotting time (in minutes) | | 120 | 16 | 119 | 18 | 20 |
| Serum prothrombin time 3 hours after venipuncture (in seconds) | | 12.9 | 47.0 | 12.0 | 43.8 | 40.2 |

The results obtained with the differential diagnostic tests in a case of PTC deficiency are presented in Table IV and Fig. 2.

The simplest differentiating test is the clotting time. The prothrombin consumption test is very helpful in studying cases of mild hemophilia with a coagulation time approaching or within normal limits. This study includes eight such patients: two patients with AHG deficiency, two with PTC deficiency, and all four patients having PTA deficiency. The one-stage prothrombin consumption test is easily adaptable to clinical use because of its simplicity, but it is less accurate than the two-stage test.

The relative incidence of AHG, PTC, and PTA deficiency is represented in Table V.

The case ratio AHG:PTC:PTA deficiency was found to be 45:6:4; the family ratio, calculated by computing each family with one or more hemophilic as a unit, was 33:4:1. It appears that AHG deficiency occurs most frequently. The case incidence of PTC deficiency (10.9 per cent) is close to the figures reported by Biggs and associates[4] and by Soulier and Larrieu[8]: among a total of sixty-eight patients the two groups of investigators found eleven cases of PTC deficiency or 16 per cent. Of the cases with positive family history we have tested and confirmed the sex-linked recessive inheritance in eight instances of AHG deficiency and in two instances of PTC deficiency.

TABLE V. RELATIVE INCIDENCE OF AHG, PTC, AND PTA DEFICIENCY

| DISEASE ENTITY | NUMBER OF CASES | PERCENTAGE CASE INCIDENCE | NUMBER OF FAMILIES | PERCENTAGE FAMILY INCIDENCE |
|---|---|---|---|---|
| AHG deficiency | 45 | 81.8 | 33 | 86.9 |
| PTC deficiency | 6 | 10.9 | 4 | 10.5 |
| PTA deficiency | 4 | 7.3 | 1 | 2.6 |
| Total | 55 | 100 | 38 | 100 |

The four cases of PTA deficiency were members of a family in which there was historical evidence of bleeders for four generations. We had the opportunity to study three boys and their mother; the other bleeders were the boys' aunt, grandfather, and great-grandmother, all straight line descendants on the maternal side. The mother had several severe hemorrhagic episodes after surgical interventions and following deliveries, but was otherwise bothered very little with hemorrhagic symptoms. Two sons bruised very easily after minor traumata; the third one had only occasional ecchymoses. The consumption of prothrombin tested with the one-stage method was always very poor: the serum prothrombin times three hours after venipuncture ranged between 14.4 and 14.6 seconds for the mother and between 15.0 and 16.1 seconds for the boys. The clotting times were either at the upper limit of normal or only slightly elevated: 22, 24, 20, and 20 minutes respectively (normal up to 20 minutes). Normal serum and $BaSO_4$ treated plasma corrected the clotting defect in all four members of the family. The mother's plasma in turn reverted to normal the defect of PTC and AHG deficiency. The clinical, genetic, and laboratory data of these four cases are very similar to the results of the original report of Rosenthal and associates.[5]

## DISCUSSION

By means of simple matching experiments it is now possible to subdivide hemophilia into three separate disease entities. The careful study of these congenital hemorrhagic diatheses will extend our knowledge of the basic mechanism of blood coagulation.

The similarity of the coagulation defect in the three conditions strongly suggests that the three deficient factors act at the same phase of the normal clotting process. The prothrombin consumption test has been interpreted as the best available index of thromboplastin activity provided the concentration

of labile factor is adequate. The uniformly poor consumption of prothrombin in AHG, PTC, and PTA deficiency indicates that these three clotting factors play an important role during thromboplastin formation. The exact mechanism of interaction among AHG, PTC, PTA, and platelets remains an enigma.

From a clinical standpoint, AHG and PTC deficiency are indistinguishable. The range of severity of hemorrhagic symptoms is quite wide in both diseases: This study includes patients of either type of deficiency with only occasional hemorrhagic episodes, as well as patients who became badly crippled after repeated hemarthroses. The hereditary pattern in both conditions is recessive and sex-linked. In their isolated report, Rothenthal and associates[5] describe PTA deficiency as a mild hemorrhagic diathesis occurring in both sexes. The present report confirms their findings. Further case studies will be necessary, however, before these characteristics can be accepted as differential diagnostic criteria.

The mainstay of our therapeutic armamentarium in all three types of hemophilia is normal plasma or blood. White and colleagues[3] have shown that the effect of transfused plasma lasts much longer in PTC deficiency than in AHG deficiency. This observation suggests that the turnover rate of PTC is slower than that of AHG. Normal serum is effective in PTC deficiency and inert in AHG deficiency. The instability of AHG in normal plasma has made it mandatory to give fresh plasma for therapeutic use. PTC is more stable to storage than AHG, as demonstrated by the effectiveness of old plasma to correct the clotting defect of PTC deficiency, in vivo and in vitro.[4] Cohn's fraction I is devoid of PTC activity. The inefficiency of this preparation in a certain number of hemophiliacs can easily be explained in the light of the recent subclassification of hemophilia: AHG deficiency responds to Cohn's fraction I, PTC deficiency remains unaffected.

## SUMMARY

Recent studies have shown that hemophilia includes three different disease entities: AHG deficiency, PTC deficiency, and PTA deficiency. Fifty-five patients whose diseases were previously diagnosed simply as hemophilia were restudied and reclassified. The clotting defect in forty-five of them was found to be due to AHG deficiency; PTC deficiency was the cause of the hemorrhagic diathesis in six patients, and the remaining four had PTA deficiency.

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# THE ANTIGLOBULIN (COOMBS) TEST AS AN AID IN THE DIAGNOSIS OF HEMOLYTIC SYNDROMES

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WHILE the antiglobulin (Coombs) test has received considerable publicity as a diagnostic aid in hemolytic anemia, no critical analysis of its limitations has been forthcoming. This study was undertaken because many of the reported results have been at variance with those observed at the Mayo Clinic.

The antiglobulin test was developed by Coombs and associates,[1] in 1945, as a method for the detection of "incomplete" antibodies in Rh sensitization. Boorman and his group[2] were the first to apply the test in the diagnosis of hemolytic anemia in adults; they reported consistently positive results in hemolytic anemia of the so-called idiopathic acquired variety. No agglutination was found in their cases of congenital spherocytosis. Others soon reported similar results,[3-7] although it was soon recognized that negative results of this test were obtained in certain cases of acquired hemolytic anemia. The nonspecificity of the test was pointed out strikingly by Wright and associates,[8] who reported positive results in multiple myeloma, thalassemia, and idiopathic thrombocytopenic purpura in which there was no evidence of hemolysis. While several of the limitations of the test have been mentioned in the literature,[9-11] we consider that these have not been stressed sufficiently.

## METHODS

Multiple direct and indirect Coombs' tests, using antiglobulin serums from as many as six different commercial sources, have been performed on the blood of twenty-one patients who had various types of hemolytic disease. Each of these patients had values for indirect serum bilirubin greater than 1.2 mg. per 100 ml. or twenty-four-hour excretion of fecal urobilinogen of 300 mg. or more or both.

In the performance of the direct antiglobulin test, the patient's erythrocytes were washed three times and a 2 per cent suspension of these washed erythrocytes was made in isotonic solution of sodium chloride. One drop of antiglobulin serum and one drop of the suspension were incubated in a water bath at 37° C. for one hour. After centrifugation at 1,000 r.p.m. for two minutes, the preparation was examined for the presence of agglutination after gentle disturbance over a concave lens.

In the indirect determination, type O erythrocytes from an Rh-positive person were exposed to the patient's serum for one hour at 37° C., washed three times, and used in the direct test as described above. When positive results were obtained in either test, determinations using serial dilutions of antiglobulin serum were carried out. The strength of agglutination was graded from 1 plus to 4 plus.

The Mayo Foundation is a part of the Graduate School of the University of Minnesota.
Received for publication, Jan. 16, 1954.
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To enhance the evaluation of positive results of Coombs' tests in idiopathic acquired hemolytic anemia, a review was made of the records in forty-six such cases seen at the clinic from 1950 to 1952, inclusive. In none of these cases was a positive family history for hemolytic disease obtained, nor was there any evidence for a primary disorder as the underlying cause.

## RESULTS

Striking discrepancies in a given test using multiple serums soon became apparent. In seven of the ten cases in which at least one serum gave positive results, one or more serums completely failed to produce agglutination. Variations in titers of agglutination of at least two dilutions were present in eight of these ten cases.

## REPORT OF CASES

CASE 1.—A 49-year-old white woman came to the clinic in June, 1952. In 1932, she had been told she was anemic. No treatment had been given until 1939, when she had received a single blood transfusion. In 1948, her erythrocyte count had decreased to 1,400,000 per cubic millimeter of blood and a diagnosis of splenic anemia had been made. Four transfusions had been required during the three months prior to her first visit to the clinic.

The family history was noncontributory. Results of examination were not remarkable except for moderate jaundice and enlargement of the spleen to four fingerbreadths below the costal margin. Laboratory studies gave the following results: Erythrocytes numbered 2,040,000, leukocytes 8,200, and platelets 187,000, per cubic millimeter of blood. Hemoglobin measured 7.5 Gm. per 100 ml. of blood. The value for serum bilirubin was zero direct and 3.0 mg. indirect. Erythrocytic fragility was within normal limits. Excretion of fecal urobilinogen was 767 mg. in twenty-four hours. Results of study of blood smears and bone marrow were consistent with the diagnosis of acquired hemolytic anemia. Splenectomy was performed on June 25, 1952.

The postoperative course was uneventful. Within two weeks the value for indirect bilirubin had decreased to 1.0 mg. and that for hemoglobin had increased to 9.8 Gm. At the time of dismissal, no further evidence of hemolyis had been noted. Results of direct antiglobulin tests are recorded in Table I.

TABLE I. DEGREE OF AGGLUTINATION IN ANTIGLOBULIN TESTS (CASE 1)

| | | SERUM SOURCE | | | | | |
|---|---|---|---|---|---|---|---|
| | DILUTION | A | B | C | D | E | F |
| Before splenectomy | Undil. | ? | + | 0 | + | + | +++ |
| | 1:2 | 0 | + | 0 | 0 | 0 | + |
| | 1:4 | 0 | 0 | 0 | 0 | 0 | 0 |
| After splenectomy | Undil. | +++ | +++ | ++ | +++ | ++ | +++ |
| | 1:2 | ++ | +++ | + | ++ | + | + |
| | 1:4 | ++ | ++ | 0 | 0 | + | 0 |
| | 1:8 | + | + | 0 | 0 | 0 | ? |
| | 1:16 | 0 | 0 | 0 | 0 | 0 | 0 |

CASE 2.—A 48-year-old white woman came to the clinic in August, 1952. She had become anemic first in 1948, at which time jaundice was noted. She had received about forty blood transfusions. In 1950, anemia recurred and twenty more transfusions were required. At this time a large spleen was found and removed. After this she did well until about a month prior to her visit to the clinic. The family history was noncontributory. Laboratory studies revealed the following values: hemoglobin 7.6 Gm.; erythrocytes 2,040,000; leukocytes 13,800 (normal differential count); reticulocytes 21.5 per cent; platelets 647,000; indirect serum bilirubin 1.58 mg. Erythrocytic fragility was increased, with hemolysis beginning at 0.75 per cent solution of sodium chloride and complete at 0.34 per cent. Excretion

of fecal urobilinogen was 206 mg. in twenty-four hours. Blood smears showed spherocytes, normoblasts, polychromasia, and Jolly bodies. Results of direct antiglobulin tests are recorded in Table II.

TABLE II. DEGREE OF AGGLUTINATION IN ANTIGLOBULIN TESTS (CASE 2)

| | SERUM | | | | | |
|---|---|---|---|---|---|---|
| | SOURCE | | | | | |
| DILUTION | A | B | C | D | E | F |
| Undil. | +++ | + | 0 | ++ | 0 | ++ |
| 1:4 | +++ | ++ | 0 | ++ | 0 | ++ |
| 1:8 | 0 | ? | 0 | + | 0 | + |

CASE 3.—A 58-year-old white man came to the clinic in April, 1952. He had been found to have chronic lymphocytic leukemia in 1949. Irradiation of the spleen at this time gave some improvement but a second course, in 1951, failed to do so. Several months before his visit to the clinic, severe anemia was found and five transfusions were given. Three days before admission, he had noted dark urine. Results of laboratory studies were as follows: erythrocytes 2,480,000; hemoglobin 6.5 Gm.; platelets 16,000; reticulocytes 5.3 per cent; leukocytes 76,000; serum bilirubin zero direct and 5.3 mg. indirect. Excretion of fecal urobilinogen was 1,343 mg. in twenty-four hours. Results of the antiglobulin test were positive.

Administration of cortisone was begun April 13, 1952, and he was given the first of eight blood transfusions. Within a week the hemoglobin had increased to 10.4 Gm. and the value for indirect bilirubin had decreased to 0.9 mg. Results of the Coombs test remained positive.

He returned in June, 1952, for follow-up study. At this time the hemoglobin measured 10.8 Gm., reticulocytes numbered 3.3 per cent, the indirect bilirubin measured 0.7 mg., and the twenty-four-hour excretion of fecal urobilinogen was 378 mg. Results of a routine Coombs test using a single serum were negative. The spleen was down four fingerbreadths and the liver was palpable. Moderate enlargement of the cervical and axillary lymph nodes was noted. Multiple antiglobulin determinations gave the results noted in Table III.

TABLE III. DEGREE OF AGGLUTINATION IN ANTIGLOBULIN TESTS (CASE 3)

| | SERUM | | | | | |
|---|---|---|---|---|---|---|
| | SOURCE | | | | | |
| DILUTION | A | B | C | D | E | F |
| Undil. | ++ | + | 0 | + | 0 | + |
| 1:2 | ++ | 0 | 0 | + | + | 0 |
| 1:4 | + | 0 | 0 | + | + | 0 |
| 1:8 | ? | 0 | 0 | 0 | 0 | 0 |

CASE 4.—A 59-year-old white man had a left lower lobectomy for bronchiectasis at the clinic in February, 1951. At a follow-up visit in November, 1951, he was found to be mildly anemic. His spleen was enlarged to five fingerbreadths below the costal margin. Results of laboratory studies were as follows: hemoglobin 10.4 Gm.; reticulocytes 4.9 per cent; serum bilirubin zero direct and 1.7 mg. indirect. Excretion of fecal urobilinogen was 448 mg. in twenty-four hours. Erythrocytic fragility was normal; results of the antiglobulin test were positive. Administration of cortisone was started but was discontinued soon after the patient left the clinic. In February, 1952, he noted pronounced fatigability. Examination revealed evidence of further hemolysis and use of cortisone was resumed. He did well until October, 1952, when he returned because of acute pneumonitis. At this time the indirect bilirubin measured 1.6 mg. and the hemoglobin 10 Gm. Multiple antiglobulin tests gave the results noted in Table IV.

TABLE IV. DEGREE OF AGGLUTINATION IN ANTIGLOBULIN TESTS (CASE 4)

| | SERUM | | | | | |
|---|---|---|---|---|---|---|
| | SOURCE | | | | | |
| DILUTION | A | B | C | D | E | F |
| Undil. | +++ | ++ | 0 | + | + | ++ |
| 1:2 | ++ | + | 0 | 0 | + | + |
| 1:4 | ++ | + | 0 | 0 | + | + |
| 1:8 | + | 0 | 0 | 0 | 0 | 0 |
| 1:16 | 0 | 0 | 0 | 0 | 0 | 0 |

CASE 5.—This patient was the premature first-born child of an Rh-negative mother and an Rh-positive father. The mother had received a blood transfusion at the age of 11. Although the child apparently was normal at birth, jaundice was apparent on the following day. The course was rapidly downhill and the infant died after signs of extreme respiratory distress. Although necropsy was not done, death was thought to be due to atelectasis and erythroblastosis fetalis. On the day of birth, the hemoglobin measured 10 Gm. and erythrocytes numbered 3,510,000. The leukocyte count disclosed 40,900 cells (not corrected for normoblasts). Normoblasts numbered 76 per 100 leukocytes and the incidence of reticulocytes was 8.8 per cent. The child was Rh-positive. Antiglobulin tests gave the results noted in Table V.

TABLE V. DEGREE OF AGGLUTINATION IN ANTIGLOBULIN TESTS (CASE 5)

| | SERUM | | | | | |
|---|---|---|---|---|---|---|
| | SOURCE | | | | | |
| DILUTION | A | B | C | D | E | F |
| Undil. | +++ | ++++ | +++ | +++ | ? | ++++ |
| 1:2 | + | ++++ | +++ | ++ | + | ++++ |
| 1:4 | + | ++++ | + | + | 0 | +++ |
| 1:8 | + | ++ | + | + | 0 | + |
| 1:16 | 0 | ++ | + | 0 | 0 | 0 |
| 1:32 | 0 | + | 0 | 0 | 0 | 0 |
| 1:64 | 0 | ? | 0 | 0 | 0 | 0 |
| 1:128 | 0 | 0 | 0 | 0 | 0 | 0 |

CASE 6.—This patient was the Rh-positive son of an Rh-negative mother and an Rh-positive father. At his birth the erythrocytes numbered 3,280,000; the hemoglobin measured 11.6 Gm. and the incidence of reticulocytes was 31.7 per cent. Jaundice developed rapidly and an exchange transfusion was given. He was dismissed six days after birth in excellent condition. Antiglobulin tests gave the results noted in Table VI.

TABLE VI. DEGREE OF AGGLUTINATION IN ANTIGLOBULIN TESTS (CASE 6)

| | SERUM | | | | | |
|---|---|---|---|---|---|---|
| | SOURCE | | | | | |
| DILUTION | A | B | C | D | E | F |
| Undil. | 0 | 0 | 0 | 0 | ++ | + |
| 1:2 | 0 | 0 | 0 | + | 0 | + |
| 1:4 | 0 | 0 | 0 | 0 | 0 | 0 |

CASE 7.—This girl was one of a pair of fraternal twins born of an Rh-negative mother and an Rh-positive father. She was Rh-positive, whereas her twin brother was Rh-negative. Results of Coombs' test on the latter were negative. At birth the patient's liver and spleen each were enlarged one fingerbreadth below the costal margins. Jaundice developed on the following day and an exchange transfusion of 250 ml. was given. The

patient was dismissed ten days after birth in good condition. Erythrocytes numbered 4,310,000 and leukocytes 12,900. Indirect serum bilirubin measured 2.2 mg. and the incidence of reticulocytes was 5.2 per cent. Antiglobulin tests gave the results noted in Table VII.

TABLE VII. DEGREE OF AGGLUTINATION IN ANTIGLOBULIN TESTS (CASE 7)

| | SERUM | | | | |
|---|---|---|---|---|---|
| | SOURCE | | | | |
| DILUTION | A | B | C | E | F |
| Undil. | +++ | ++ | +++ | 0 | ++ |
| 1:2 | +++ | + | ++ | 0 | +++ |
| 1:4 | ++ | + | + | 0 | + |
| 1:8 | + | 0 | + | 0 | 0 |
| 1:16 | 0 | 0 | ? | 0 | 0 |

Among the twenty-one patients in this series, eleven were diagnosed as having idiopathic acquired hemolytic anemia. In only four of these patients were positive results obtained in antiglobulin tests, even with the use of six serums. Review of the records showed positive results in routine Coombs' tests performed with one serum in twenty-three of the forty-six previously mentioned patients who had idiopathic acquired hemolytic anemia.

## COMMENT

The usefulness of any laboratory determination from the clinical standpoint depends not only on what the test tells the clinician but also on the reliability that can be placed on the result of any given test. It can be readily seen from this study that the antiglobulin test is open to serious criticism on both counts.

The variability in strength of Coombs' serums has been pointed out by a number of workers.[9, 11] This has been demonstrated strikingly by our study, in which all of the preparations used were marketed commercially as high-potency antiglobulin serums. The technical problem imposed by this lack of uniformity is difficult to ignore in a consideration of the validity of so-called negative Coombs' tests. One is soon confronted with the pertinent question of "How many serums are enough?" Throughout this study, one serum (C) tended to be the least effective in agglutination; in addition, considerable variation was noted in strength of reactions from case to case. This would suggest that factors other than mere "potency" may be involved and that the use of positive controls may not be valid in assessing the efficacy of a given serum.

The observation by van Loghem and associates[12] of prozone phenomena in antiglobulin tests points out another difficulty that may invalidate the results of a given test unless serial dilutions are carried out routinely. While this study was not designed to demonstrate this phenomenon, a number of instances were observed in which increased agglutination occurred with the use of diluted serums.

That cold agglutinins may cause false positive antiglobulin reactions even when the samples of blood are kept at 37° C. has been recognized by Dacie

and de Gruchy[13] and confirmed by Young and associates.[11] The significance of this observation in the evaluation of results reported by various workers is not clear, since tests to determine the presence of these apparently frequently occurring agglutinins usually have not been reported. Witebsky[14] has reported that the Donath-Landsteiner antibody also may sensitize erythrocytes to antiglobulin serums.

Other sources of error that may tend to invalidate the result of a given test are largely technical in nature and can be prevented by a moderate amount of care. These include such factors as inadequate washing of erythrocytes, inaccurate preparation of suspensions of erythrocytes, which makes it difficult to determine the presence or absence of agglutination, and centrifugation at too great a speed.

The question as to what the results of a given test tell the clinician is extremely difficult to answer in view of the current state of knowledge as to the mechanism of the Coombs reaction and the pathogenesis of the hemolytic disease process itself. In our study, results of antiglobulin tests have been positive in less than half of the patients who were diagnosed as having acquired hemolytic anemia. It is widely recognized that no change may occur in the results of Coombs' test even after a remission is obtained by splenectomy or steroid therapy. In Case 1 of our series, a great increase took place in the strength of agglutination after operation and with apparent cessation of the hemolytic process. We, as well as others,[9] have found no correlation between the degree of agglutination and the severity of the hemolytic process. We have encountered no cases of congenital spherocytosis in which positive Coombs' reactions were present, although this has been reported by others.[5, 8]

## SUMMARY AND CONCLUSIONS

While the limitations of the antiglobulin (Coombs) test have been mentioned in the literature, they have not been stressed sufficiently. Variation in potency of the available commercial serums is great, which places a great limitation on the validity of any given determination, even when multiple serums are used.

Other factors, such as possible prozone phenomena, cold agglutinins, the Donath-Landsteiner antibody, and numerous technical difficulties, must be weighed in each determination.

Negative results of Coombs' test have no significance in the differentiation of acquired and congenital forms of hemolytic disease. Positive results are of value.

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# LEVELS OF 17-HYDROXYCORTICOSTEROIDS IN BODY FLUIDS

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## INTRODUCTION

THE concentration of adrenal corticosteroids in the plasma and urine of normal subjects and of patients with various disorders has been estimated by several techniques. No reports of the presence of corticosteroids in body fluids other than blood or urine have appeared, however. In this paper determinations of the levels of endogenous 17-hydroxycorticosteroids in the spinal fluids of normal subjects and of patients with various disease states are presented. In addition, the concentration of 17-hydroxycorticosteroids in pleural, ascitic, and pericardial fluids from patients with various pathologic conditions is reported.

## MATERIALS AND METHODS

Body fluids, other than blood, were collected without preservative or anticoagulant. Heparin (4 mg. per 30 ml. of whole blood) was used as an anticoagulant in blood. The plasma values reported were determined on blood drawn at the same time as the other body fluids which are reported.

The free 17-hydroxycorticosteroids (17, 21-dihydroxy-20-ketosteroids) were determined by a modification[1] of the method of Nelson and Samuels.[2] Conjugated steroids are not measured by this procedure.

## RESULTS

Table I records the levels of 17-hydroxycorticosteroids in the spinal fluids of subjects without any apparent disease and of patients with various clinical conditions. The levels in the spinal fluids are compared with plasma levels of 17-hydroxycorticosteroids in some of these subjects.

None of the spinal fluids from seven normal subjects contained demonstrable 17-hydroxycorticosteroids. Since the analytic method is reliable for concentrations of 2 $\mu$g per 100 ml. or higher, the spinal fluids must have contained less than this. Simultaneous plasma levels were within the normal limits.[3] In addition, in five of the patients without diseases of the central nervous system, no demonstrable levels of 17-hydroxycorticosteroids were present in the spinal fluid. The same was true of two of five patients with thromboses of cerebral vessels (Cases 13 and 14). One of these (Case 14) was

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This investigation was supported in part by research grants from the National Institutes of Health, United States Public Health Service, and in part by a contract (DA-49-007-MD-136) between the University of Utah and the Department of the Army.

Received for publication, Jan. 11, 1954.

*Scholar in Cancer Research of the American Cancer Society (1953 to 1956). The work reported in this paper was performed during the tenure of Research Fellowships of the National Institutes of Health (1951-1952) and the Dazian Foundation (1952-1953).

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in a terminal state and his plasma 17-hydroxycorticosteroid level was elevated (35 μg per 100 ml.), as is usually seen in terminal patients,[4] yet no 17-hydroxycorticosteroids were found in his spinal fluid. In two additional patients without central nervous system diseases, the levels of 17-hydroxycorticosteroids in the spinal fluids were within the range (2 μg per 100 ml. of fluid) of the lower limit of the method.

TABLE I. RELATION OF 17-HYDROXYCORTICOSTEROID LEVELS IN SPINAL FLUID TO PLASMA LEVELS IN NORMAL AND DISEASED SUBJECTS

| CASE NO. | 17-OH STEROIDS IN SPINAL FLUID (μG PER 100 ML.) | 17-OH STEROIDS IN PLASMA (μG PER 100 ML.) | TOTAL PROTEIN IN SPINAL FLUID (MG. PER 100 ML.) | CELLS PER C. MM. IN SPINAL FLUID | CLINICAL CONDITION |
|---|---|---|---|---|---|
| 1 | 0 | 8 | 35 | 0 | Normal |
| 2 | 0 | 17 | 26 | 0 | Normal |
| 3 | 0 | 12 | 30 | | Normal |
| 4 | 0 | 9 | | | Normal |
| 5 | 0 | 11 | 32 | 0-1 | Normal |
| 6 | 0 | 13 | 25 | | Normal |
| 7 | 0 | 19 | | | Normal |
| 8 | 0 | 12 | 40 | 0 | Psychoneurosis |
| 9 | 0 | 10 | | | Hypertension |
| 10 | 0 | 15 | 41 | 0 | Infectious hepatitis |
| 11 | 0 | 13 | 64 | | Cirrhosis of the liver |
| 12 | 0 | 15 | 35 | 0 | Diabetes mellitus |
| 13 | 0 | | 50 | 0 | Diabetes mellitus and cerebral vascular accident |
| 14 | 0 | 35 | 40 | 1 | Cerebral vascular thrombosis. Patient dying |
| 15 | 2 | 16 | 46 | 0 | Cirrhosis of the liver<br>Latent syphilis<br>Congestive heart failure |
| 16 | 2 | | 68 | 0 | Abscess of lung |
| 17 | 3 | | 26 | | Cerebral vascular accident |
| 18 | 3 | | | | Spinal fluid obtained shortly before death from cerebral vascular accident |
| 19 | 7 | | 88 | 0 | Cerebral vascular accident |
| 20 | 11 | 14 | 20 | 2 mononuclear cells | Ca of larynx<br>TBC of lung<br>Convulsions |
| 21 | 4 | | | | Acute poliomyelitis |
| 22 | 9 | | 135 | 250 mononuclear cells | Acute poliomyelitis |
| 23 | 6 | | 126 | 20 mononuclear cells | Acute poliomyelitis |
| 24 | 6 | 34 | 118 | 210 mononuclear cells | Acute poliomyelitis |
| 25 | 10 | 60 | 180 | 400 PMN | Pneumococcal meningitis |
| 26 | 15 | | | | Pneumococcal meningitis |

In ten patients the levels of 17-hydroxycorticosteroids in the spinal fluid were definitely elevated. Four of these patients had acute poliomyelitis and two had pneumococcal meningitis. Three patients had had cerebrovascular accidents; although no inflammatory central nervous system disease was demonstrated in the remaining patient, he had had multiple convulsions before the spinal fluid was obtained. In five of the seven patients with elevated

levels of 17-hydroxycorticosteroids in the spinal fluid, the spinal fluid protein was also elevated. In addition, in some of these patients there were also abnormal numbers of leukocytes in the spinal fluids.

Three normal subjects were each given 150 mg. of hydrocortisone or cortisone acetate orally in order to raise the plasma levels of 17-hydroxycorticosteroids.[5] Spinal fluid was obtained one to two hours after the ingestion of the steroids. Although the plasma levels reached great heights (Table II), no significant amounts of 17-hydroxycorticosteroids were found in the spinal fluids of these subjects.

TABLE II. 17-HYDROXYCORTICOSTEROID LEVELS IN THE PLASMA AND SPINAL FLUID OF THREE NORMAL SUBJECTS FOLLOWING THE ORAL ADMINISTRATION OF ADRENAL STEROIDS

| DOSAGE OF STEROID ADMINISTERED | HOURS AFTER STEROID INGESTION | PLASMA 17-OH CORTICOSTEROIDS μG PER 100 ML. | SPINAL FLUID 17-OH CORTICOSTEROIDS μG PER 100 ML. |
|---|---|---|---|
| 150 mg. cortisone acetate | 1 | 101 | 0 |
| 150 mg. hydrocortisone acetate | 2 | 33 | 3 |
| 150 mg. cortisone acetate | 2 | 72 | 0 |

Tables III and IV give the levels of 17-hydroxycorticosteroids in twenty-four samples of pleural, ascitic, and pericardial fluids of seventeen patients with various clinical conditions. In five patients the levels of 17-hydroxycorticosteroids in the fluids, all of which appeared to be transudates, were below 3 μg per 100 ml. Such values must be considered of equivocal significance for technical reasons.

In two patients (Cases 33 and 42) the levels observed in these fluids, both of which were of inflammatory character, were higher than simultaneous

TABLE III. 17-HYDROXYCORTICOSTEROID LEVELS IN THE PLEURAL FLUID AND PLASMA IN PATIENTS WITH VARIOUS DISEASE STATES

| CASE NO. | 17-OH STEROIDS IN PLEURAL FLUID (μG PER 100 ML.) | 17-OH STEROIDS IN PLASMA (μG PER 100 ML.) | PROTEIN IN PLEURAL FLUID (GM. PER 100 ML.) | CHARACTER OF AND CELLS IN PLEURAL FLUID PER C. MM. | CLINICAL CONDITION AND REMARKS |
|---|---|---|---|---|---|
| 11 | 8 | | 2.2 | | Cirrhosis of the liver |
| | 6 | 13 | 4.4 | 250,000 RBC | One month later |
| 28 | 13 | | | 2,000 RBC<br>6,000 WBC | Advanced lymphosarcoma with bronchopneumonia |
| 29 | 10 | | 3.2 | 1,000 WBC | Carcinoma of the breast |
| | 7 | | 3.4 | | Values six months later |
| 30 | 10 | 19 | | 2,000 PMN | Infarction of the lung |
| 31 | 0 | 20 | 4.2 | 6,000 RBC<br>580 Lymph | Subacute leukemia |
| | 2 | | | 80,000 RBC<br>700 Lymph | Values two weeks later |
| 32 | 7 | | | | Hodgkin's disease |
| | 10 | 18 | | | Values ten days later |
| 33 | 16 | 12 | | Gross pus | Empyema |
| 34 | 2 | | 1.3 | 830 RBC | Nephrotic syndrome |
| 35 | 10 | 17 | | | Carcinoma of the lung |

values in the plasma. However, there seems to be no obvious relation between the total protein content of these fluids and their levels of 17-hydroxycorticosteroids. Neither is there any apparent relation between the number or kind of cells and the steroid levels in the fluids.

TABLE IV. 17-HYDROXYCORTICOSTEROID LEVELS IN THE ASCITIC AND PERICARDIAL FLUIDS AND IN THE PLASMA OF PATIENTS WITH VARIOUS DISEASE STATES

| CASE NO. | 17-OH STEROIDS IN ASCITIC FLUID (μG PER 100 ML.) | 17-OH STEROIDS IN PLASMA (μG PER 100 ML.) | PROTEIN IN ASCITIC FLUID (GM. PER 100 ML.) | CELLS PER MM. | CLINICAL CONDITION |
|---|---|---|---|---|---|
| 11 | 6 | 11 | 3.7 | 40,000 RBC | Cirrhosis of the liver and peritonitis |
| 28 | 0 | 6 | 2.0 | | Advanced lymphosarcoma |
| 36 | 8 | 9 | 3.1 | 14,800 RBC and WBC | Myxedema and congestive heart failure |
| 37 | 7 | | 1.1 | | Cirrhosis of the liver |
| 38 | 9 | | 0.9 | | Cirrhosis of the liver |
| 39 | 4 | 15 | | 45,000 RBC 1,800 WBC | Carcinoma of the stomach and cirrhosis of the liver |
| 40 | 2 | | | | Cirrhosis of the liver |
| 41 | 0 | 13 | | | Carcinoma and cirrhosis of the liver |
| | 2 | | | | Values one month later |

*Pericardial Fluid*

| CASE NO. | 17-OH STEROIDS IN PERICARDIAL FLUID (μG PER 100 ML.) | 17-OH STEROIDS IN PLASMA (μG PER 100 ML.) | PROTEIN IN PERICARDIAL FLUID (GM. PER 100 ML.) | CHARACTER OF FLUID | CLINICAL CONDITION |
|---|---|---|---|---|---|
| 42 | 16 | 9 | | Grossly bloody | Acute lupus erythematosus |
| 43 | 5 | 16 | 1.2 | | Acute rheumatic fever |

No 17-hydroxycorticosteroids could be demonstrated in saliva collected from several normal subjects nor in gastric juice from normal subjects and patients with peptic ulcers.

## DISCUSSION

In normal subjects, no 17-hydroxycorticosteroids were demonstrated in the spinal fluid in contrast to the quantitatively measurable amounts in plasma. It would appear from the data presented that, under normal circumstances, there is a definite barrier to the passage of 17-hydroxycorticosteroids into the spinal fluid. In pathologic states, and especially in those in which the meninges are inflamed, the barrier is broken down and 17-hydroxycorticosteroids appear in the spinal fluid. The situation is analogous to that seen following the administration of penicillin. In normal subjects very little penicillin appears in the spinal fluid; on the other hand, when meningeal inflammation is present, penicillin appears in the spinal fluid in large amounts.

Significant 17-hydroxycorticosteroid concentrations were present in most pleural, ascitic, and pericardial fluids. With the exception of two cases, these

concentrations were lower than the plasma levels. In both exceptions the serosal membranes were inflamed. Three explanations for the lower values in the extravascular fluids appear logical. Since these are body fluids accumulated in abnormal volume, their components, including the 17-hydroxycorticosteroids, probably do not exchange rapidly with those in the circulating plasma. The samples were, in most cases, taken in the early morning. Other studies[3] have shown that the levels of 17-hydroxycorticosteroids in the plasma fall progressively during the day. At this time, the levels in the accumulated extravascular fluids may not have reached equilibrium with the higher morning levels from the low levels which existed during the preceding day. Another possible explanation is that the concentration of unbound 17-hydroxycorticosteroids in equilibrium with those bound loosely to plasma protein[6] is the same in plasma as those found in these fluids. This would be the only portion which would be freely diffusible, the protein-bound steroids serving simply as a reservoir. A last possibility is that these steroids are metabolized in the fluids and tissues. Wilson and associates[7] have shown that 17-hydroxycorticosteroids are metabolized in inflamed joints coincident with relief of symptoms. Since under such conditions the concentration of 17-hydroxycorticosteroids increases in the extravascular fluids against a normal level in the plasma, it would appear that some factor associated with inflammation accelerates diffusion of these steroids from the blood vessels. That this occurs in the fluid associated with the inflammatory area while other fluids in the same patient are normal, is shown in Cases 11 and 28. It appears that this is one mechanism by which the supply of steroid is adjusted to local need.

Concentrations in normal interstitial fluids were not determined in this study. It would be desirable to obtain such values if adequate amounts of fluid could be obtained. Thus far, no such opportunity has arisen.

## SUMMARY

There were no 17-hydroxycorticosteroids found in detectable concentrations in spinal fluids from normal subjects, even after high plasma levels had been induced by the oral administration of cortisone or hydrocortisone. Pathologic spinal fluids contained measurable 17-hydroxycorticosteroids in concentrations that were less than those in the plasma, particularly when meningeal inflammation was present. Most pleural, ascitic, and pericardial fluids contained 17-hydroxycorticosteroids; these levels, too, were usually lower than the simultaneously sampled plasma levels of 17-hydroxycorticosteroids. There appeared to be some correlation between the degree of inflammation and the concentration of 17-hydroxycorticosteroids in the extravascular fluids.

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# THE EFFECT OF HEXAMETHONIUM BROMIDE ON THE CIRCULATORY AND METABOLIC RESPONSE TO INSULIN HYPOGLYCEMIA IN MAN

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IT IS generally accepted that insulin hypoglycemia stimulates sympathetic activity and adrenaline secretion, thereby mobilizing glycogen from the liver and so restoring the blood glucose level to normal,[1] and recently it has been shown that increased amounts of adrenaline derivatives can be recovered from the urine following insulin hypoglycemia.[2] Ganglion blocking agents given with insulin should, therefore, prolong hypoglycemia by preventing the release of adrenaline, and such an effect has been reported in both animals and man.[3, 4] On the other hand, blood glucose returns to normal promptly after insulin administration in splanchnicectomized cats,[5] and observations of hepatic glucose output following insulin in man, using the hepatic vein catheterization technique, have suggested that factors other than adrenaline release may be involved.[6] Hepatic vein catheterization has therefore been used to estimate the response of the liver to hexamethonium and insulin administered simultaneously, and results have been compared with those previously obtained after insulin,[6] hexamethonium,[7] and adrenaline.[8]

## METHODS

The methods employed were similar to those reported previously.[7] A radiopaque nylon catheter was passed into a hepatic vein of fasting subjects under fluoroscopic control. Bromsulphalein (B.S.P.) was infused at a constant rate into a peripheral vein; after a period of stabilization, at least three control samples of blood were withdrawn simultaneously from the hepatic vein and brachial artery. Hexamethonium bromide (C6) (1 mg. per kilogram body weight) and soluble insulin (0.1 unit per kilogram body weight) were simultaneously injected intramuscularly and intravenously, respectively. Further hepatic vein and arterial blood samples were then withdrawn at 5, 15, 30, 45, 60, 80, 100, and 120 minutes and analyzed for B.S.P. glucose, lactic acid, and oxygen unsaturation.[7] Estimated splanchnic blood flow (E.S.B.F.), hepatic glucose output (H.G.O.), splanchnic oxygen consumption, and splanchnic vascular resistance were calculated by methods reported previously.[7] Mean blood pressure was expressed as half the sum of the systolic and diastolic values obtained by auscultation.

The twelve subjects had no known hepatic disease or disturbance of carbohydrate metabolism and were studied during the course of catheterization for other conditions.

## RESULTS

*General Effects.*—The procedure was well tolerated by all subjects. They invariably passed from drowsiness to a peaceful sleep from which they could be

From the Department of Medicine, Postgraduate Medical School of London.
Received for publication, Jan. 18, 1954.

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easily wakened. The tremor, anxiety, and restlessness of hypoglycemia were not observed and no subject became comatose. Seven subjects sweated copiously, a similar proportion to those given insulin alone in the same dosage.[6]

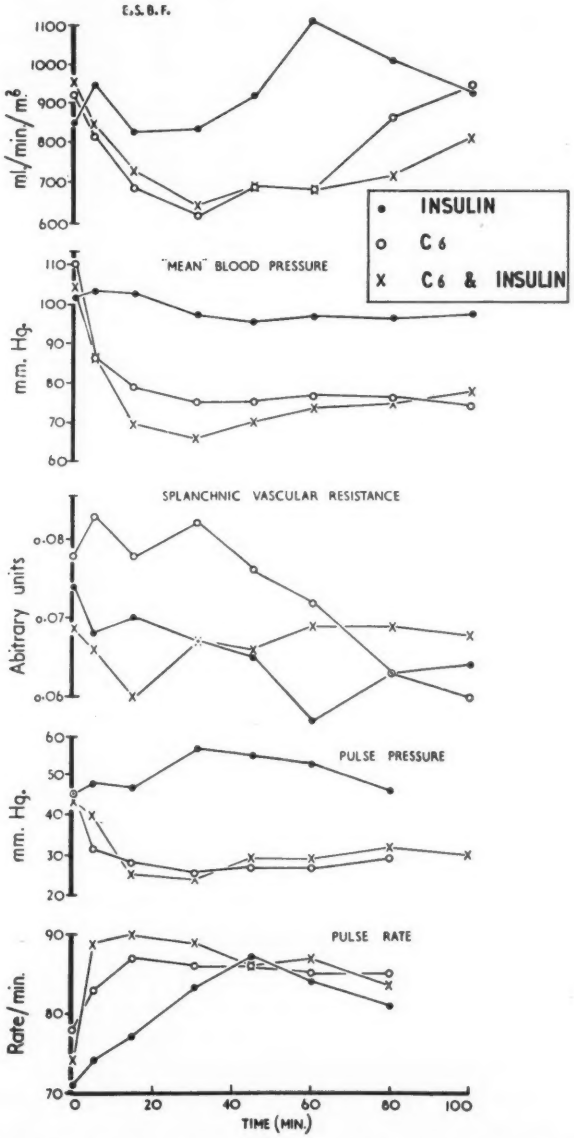


Fig. 1.—The effects of insulin, hexamethonium, and hexamethonium and insulin on pulse rate, pulse pressure, splanchnic vascular resistance, mean blood pressure, and E.S.B.F. "Mean" results for:

insulin (0.1 unit per kilogram intravenously)
● ——— ● ——— ● (14 observations)
hexamethonium (1 mg. per kilogram intramuscularly)
● ——— ● ——— ● (16 observations)
hexamethonium (1 mg. per kilogram intramuscularly) and insulin (0.1 unit per kilogram intravenously)
X ——— X ——— X (12 observations)

TABLE I. EFFECT OF HEXAMETHONIUM AND INSULIN ON E.S.B.F.

| SUBJECT | AGE | SEX | E.S.B.F. ML. PER MIN. PER SQUARE METER — MINUTES AFTER C6 AND INSULIN 0 | 5 | 15 | 30 | 45 | 60 | 80 | 100 | 120 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| J. C. | 59 | M | 1120 | 950 | 1050 | 850 | 710 | 860 | 1050 | 680 | 790 |
| G. W. | 32 | F | 1015 | – | 740 | 810 | 790 | 715 | 895 | 850 | 710 |
| W. R. | 67 | M | 730 | 730 | 490 | 400 | 460 | 460 | 460 | 520 | 590 |
| G. C. | 42 | M | 1090 | 1090 | 980 | 450 | 450 | 460 | 320 | – | – |
| D. C. | 51 | M | 1070 | 810 | 810 | 420 | 470 | 410 | 340 | 430 | 340 |
| C. B. | 62 | M | 1020 | 1035 | 710 | 510 | 630 | 670 | 670 | 1020 | 1410 |
| S. L. | 33 | M | 965 | 765 | 765 | 845 | 860 | 745 | 655 | 575 | – |
| M. W. | 50 | F | 830 | 1030 | 805 | 890 | 970 | 895 | – | 1550 | – |
| R. H. | 53 | F | 980 | 635 | 430 | 445 | 660 | 595 | 1035 | 740 | 1060 |
| W. G. | 27 | M | 1050 | 665 | 665 | 615 | 550 | 625 | 710 | 585 | 595 |
| F. R. | 45 | F | 1010 | 695 | 625 | 815 | 1010 | 970 | 935 | 985 | – |
| W. B. | 56 | M | 645 | 715 | 730 | 595 | 670 | 770 | 695 | 660 | 420 |
| | Mean | | 960 | 850 | 735 | 635 | 685 | 680 | 715 | 810 | 745 |
| | S.E. | | ±43 | ±58 | ±50 | ±56 | ±56 | ±52 | ±78 | ±110 | ±110 |

TABLE II. EFFECT OF HEXAMETHONIUM AND INSULIN ON ARTERIAL GLUCOSE

| SUBJECT | AGE | SEX | ARTERIAL GLUCOSE CONCENTRATION MG. PER 100 ML. — MINUTES AFTER C6 AND INSULIN 0 | 5 | 15 | 30 | 45 | 60 | 80 | 100 | 120 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| J. C. | 59 | M | 85 | 83 | 62 | 50 | 46 | 63 | 73 | 89 | 90 |
| G. W. | 32 | F | 68 | 61 | 34 | 41 | 47 | 54 | 59 | 71 | 71 |
| W. R. | 67 | M | 85 | 78 | 53 | 40 | 53 | 55 | 55 | 58 | 68 |
| G. C. | 42 | M | 96 | 98 | 62 | 55 | 52 | 47 | 76 | 87 | 92 |
| D. C. | 51 | M | 90 | 83 | 68 | 68 | 60 | 60 | 68 | 83 | 81 |
| C. B. | 62 | M | 80 | 77 | 49 | 43 | 79 | 75 | 85 | 96 | 103 |
| S. L. | 33 | M | 69 | 59 | 47 | 18 | 18 | 30 | 49 | 58 | – |
| M. W. | 50 | F | 77 | 68 | 54 | 47 | 47 | 34 | 34 | 40 | 40 |
| R. H. | 53 | F | 79 | 72 | 47 | 30 | 24 | 30 | 42 | 60 | 70 |
| W. G. | 27 | M | 83 | 78 | 59 | 45 | 49 | 60 | 75 | 75 | 79 |
| F. R. | 45 | F | 86 | 76 | 61 | 39 | 33 | 45 | 51 | 60 | – |
| W. B. | 56 | M | 67 | 71 | 57 | 45 | 40 | 60 | 66 | 72 | 67 |
| | Mean | | 80 | 75 | 54 | 43 | 46 | 51 | 61 | 70 | 79 |
| | S.E. | | ±2.6 | ±3.0 | ±2.6 | ±3.5 | ±4.7 | ±4.1 | ±4.4 | ±4.7 | ±5.3 |

*Circulatory Effects* (Table I, Fig. 1).— Pulse rate rose after hexamethonium and insulin. The rate of rise was more rapid, however, than with insulin alone and the maximum increase occurred at fifteen minutes, compared with the slower rise to a maximum at forty-five minutes after insulin.

"Mean" blood pressure and pulse pressure fell in a manner similar to that after hexamethonium alone. The rise in pulse pressure, which occurs thirty minutes after the administration of insulin, was not observed.

E.S.B.F. initially was similar to that reported previously,[6-8] and fell in a similar manner to that after hexamethonium alone, reaching its lowest level at thirty minutes, and subsequently rising slightly. The rise in E.S.B.F., which occurs sixty minutes after insulin administration, was not observed.

Splanchnic vascular resistance did not change significantly from the basal level. This is similar to the response during the first sixty minutes after hexamethonium alone, and in contrast to the fall observed at sixty minutes after insulin alone.

PULSE PRESSURE, AND SPLANCHNIC VASCULAR RESISTANCE

| PULSE PRESSURE (MM. HG) | | | | | | | | | SPLANCHNIC VASCULAR RESISTANCE ARBITRARY UNITS | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| MINUTES AFTER C6 AND INSULIN | | | | | | | | | MINUTES AFTER C6 AND INSULIN | | | | | | | | |
| 0 | 5 | 15 | 30 | 45 | 60 | 80 | 100 | 120 | 0 | 5 | 15 | 30 | 45 | 60 | 80 | 100 | 120 |
| 48 | 50 | 20 | 20 | 20 | 20 | 25 | 22 | 25 | .055 | .051 | .037 | .049 | .062 | .051 | .041 | .065 | .056 |
| 41 | 35 | 38 | 33 | 34 | 32 | 30 | 34 | 30 | .071 | – | .064 | .062 | .067 | .072 | .059 | .065 | .078 |
| 48 | 56 | 26 | 18 | 30 | 26 | 26 | 32 | 40 | .080 | .069 | .080 | .090 | .065 | .067 | .057 | .064 | .061 |
| 40 | 35 | 15 | 15 | 20 | 30 | 25 | 30 | 35 | .056 | .041 | .031 | .071 | .081 | .073 | .126 | – | – |
| 48 | 40 | 22 | 25 | 30 | 25 | 30 | 20 | 20 | .066 | .066 | .051 | .079 | .089 | .103 | .114 | .082 | .103 |
| 52 | 60 | 32 | 15 | 27 | 25 | 20 | 25 | 38 | .060 | .063 | .070 | .074 | .065 | .062 | .051 | .053 | .039 |
| 41 | 40 | 40 | 40 | 46 | 40 | 50 | 40 | – | .059 | .070 | .074 | .056 | .050 | .056 | .066 | .083 | – |
| 54 | 30 | 24 | 24 | 24 | 30 | 28 | 34 | 34 | .098 | .064 | .059 | .049 | .049 | .066 | – | .042 | – |
| 39 | 30 | 14 | 20 | 18 | 24 | 30 | 30 | 30 | .061 | .066 | .056 | .078 | .057 | .068 | .041 | .063 | .048 |
| 38 | 36 | 30 | 32 | 34 | 38 | 36 | 30 | 32 | .048 | .072 | .056 | .068 | .088 | .081 | .075 | .086 | .090 |
| 47 | 38 | 28 | 30 | 42 | 40 | 50 | – | – | .074 | .086 | .087 | .064 | .055 | .059 | .068 | .063 | – |
| 29 | 24 | 15 | 20 | 28 | 32 | 32 | 30 | 30 | .104 | .078 | .057 | .064 | .061 | .065 | .066 | .077 | .108 |
| 44 | 39 | 25 | 24 | 29 | 29 | 32 | 30 | 31 | .069 | .066 | .060 | .067 | .066 | .069 | .069 | .068 | .070 |
| ±2.0 | ±3.1 | ±2.5 | ±2.3 | ±2.2 | ±1.9 | ±2.7 | ±1.7 | ±1.9 | ±.005 | ±.004 | ±.005 | ±.004 | ±.004 | ±.004 | ±.008 | ±.004 | ±.010 |

CONCENTRATION, HEPATIC GLUCOSE OUTPUT, AND SPLANCHNIC OXYGEN CONSUMPTION

| HEPATIC GLUCOSE OUTPUT MG. PER MIN. PER SQUARE METER | | | | | | | | | SPLANCHNIC OXYGEN CONSUMPTION ML. PER MIN. PER SQUARE METER | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| MINUTES AFTER C6 AND INSULIN | | | | | | | | | MINUTES AFTER C6 AND INSULIN | | | | | | | | |
| 0 | 5 | 15 | 30 | 45 | 60 | 80 | 100 | 120 | 0 | 5 | 15 | 30 | 45 | 60 | 80 | 100 | 120 |
| 142 | 63 | 126 | 68 | 43 | 43 | 37 | –17 | –43 | 53 | 45 | 62 | 40 | 25 | 45 | 42 | 28 | 31 |
| 122 | – | 89 | 150 | 186 | 40 | 49 | 56 | 43 | 39 | – | 54 | 42 | 31 | 27 | 35 | 38 | 29 |
| 154 | 149 | 268 | 383 | 625 | 580 | 446 | 300 | 72 | 33 | 41 | 40 | 26 | 19 | 27 | 34 | 28 | 36 |
| 164 | 316 | – | 227 | 274 | 148 | 61 | – | – | 89 | 82 | 96 | 59 | 52 | 43 | 17 | – | – |
| 122 | 81 | 60.5 | 32 | 51 | 42 | 34 | 44 | 35 | 59 | – | – | 69 | 32 | 27 | 22 | 40 | 33 |
| 113 | 94 | 129 | 124 | 186 | 188 | 149 | 327 | 254 | 48 | – | 58 | 48 | 45 | 53 | 50 | 63 | 88 |
| 135 | 42 | 30 | 30 | 71 | 52 | 0 | 17 | – | 45 | – | 34 | 39 | 38 | 24 | 15 | 24 | – |
| 58 | 57 | 52 | 58 | 63 | 0 | – | 124 | – | 43 | 53 | 46 | 67 | 61 | 48 | – | 58 | – |
| 150 | 51 | 30 | 78 | 82 | 78 | 140 | 45 | 79 | 52 | 47 | 39 | 31 | 49 | 49 | 87 | – | 45 |
| 66 | 33 | 87 | 86 | 66 | 69 | 57 | 44 | 57 | 42 | 28 | 28 | 24 | 23 | 25 | 25 | 21 | 24 |
| 63 | 87 | 113 | 0 | 60 | 53 | 107 | 108 | – | 66 | 44 | 38 | 40 | 45 | 50 | 33 | 72 | – |
| 96 | 29 | 40 | 60 | 90 | 173 | 90 | 49 | 23 | 12 | 32 | – | 48 | 33 | 36 | – | 20 | 11 |
| 116 | 91 | 103 | 103 | 142 | 114 | 104 | 116 | 59 | 48 | 55 | 48 | 44 | 38 | 38 | 41 | 46 | 43 |
| ±10.8 | ±17.8 | ±19.4 | ±22.7 | ±34.6 | ±34.4 | ±28.8 | ±34.0 | ±30.5 | ±5.4 | ±6.5 | ±4.2 | ±4.4 | ±3.7 | ±3.3 | ±6.9 | ±6.1 | ±7.7 |

*Metabolic Effects* (Table II, Fig. 2).—Arteriovenous oxygen difference rose following administration of hexamethonium and insulin. A similar response occurred after hexamethonium alone. Since blood flow fell to the same extent, there was no over-all change in calculated splanchnic oxygen consumption. Insulin causes a rise in splanchnic oxygen consumption at sixty minutes, and this value is significantly different from that obtained sixty minutes after hexamethonium and insulin.

Arterial glucose concentration fell following the administration of hexamethonium and insulin to levels which did not differ significantly from those obtained by administration of insulin alone. The blood glucose then rose in a similar manner and returned to normal levels during the period of observations. Hexamethonium does not alter arterial glucose concentration.

Basal hepatic glucose output was identical with that obtained in previous studies using insulin[6] and hexamethonium.[7] There was a slight fall following administration of hexamethonium and insulin, but this was less than that

obtained with insulin alone. The fall after insulin administration is followed by a marked rise in HGO, which did not occur with hexamethonium and insulin. Hexamethonium alone does not alter H.G.O.

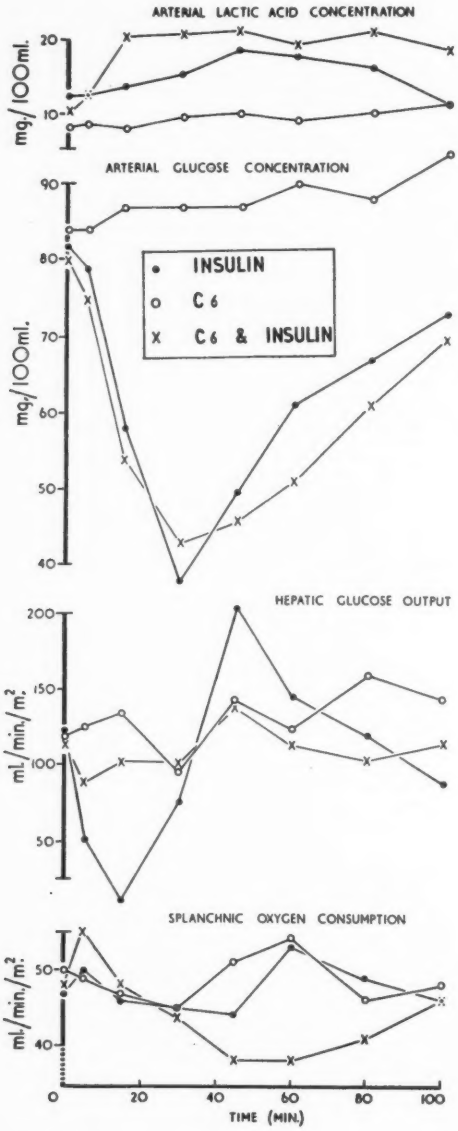


Fig. 2.—The effect of insulin, hexamethonium, and hexamethonium and insulin on splanchnic oxygen consumption, hepatic glucose output, arterial glucose concentration, and arterial lactic acid concentration. Mean results for:—

insulin (0.1 unit per kilogram intravenously)

● ——— ● ——— ● (14 observations)

hexamethonium (1 mg. per kilogram intramuscularly)

● ——— ● ——— ● (16 observations)

hexamethonium (1 mg. per kilogram intramuscularly) and insulin (0.1 unit per kilogram intravenously)

X ——— X ——— X (12 observations)

Arterial lactic acid concentration (Table III) was estimated in eleven of the twelve subjects and rose after administration of hexamethonium and insulin. The rise was sustained, was greater, and occurred earlier than that seen after insulin alone. Lactic acid was estimated on arterial, as opposed to the venous, samples used with insulin alone. There is no significant rise in arterial lactic acid concentration following hexamethonium alone.

TABLE III. EFFECT OF HEXAMETHONIUM AND INSULIN ON ARTERIAL LACTIC ACID CONCENTRATION
(MILLIGRAMS PER 100 ML.)

| NO. OF SUBJECTS | | TIME AFTER HEXAMETHONIUM AND INSULIN (MINUTES) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 0 | 5 | 15 | 30 | 45 | 60 | 80 | 100 | 120 |
| 11 | Mean | 10.5 | 13.0 | 20.0 | 20.4 | 21.3 | 18.9 | 21.5 | 17.9 | 11.9 |
| | S.E. | ±2.8 | ±2.8 | ±2.8 | ±2.3 | ±3.5 | ±2.9 | ±3.5 | ±2.3 | ±2.1 |

The circulatory and metabolic effects of hexamethonium, insulin, hexamethonium and insulin, and adrenaline are summarized in Table IV.

TABLE IV. COMPARISON OF EFFECTS SHOWING CHANGE FROM BASAL LEVELS AT FORTY-FIVE MINUTES

| | C6 | INSULIN | C6 AND INSULIN | ADRENALINE |
|---|---|---|---|---|
| Pulse rate | Increase | Increase | Increase | Increase |
| "Mean" blood pressure | Decrease | No change | Decrease | No change |
| Pulse pressure | Decrease | Slight increase | Decrease | Increase |
| Splanchnic blood flow | Decrease | Increase | Decrease | Increase |
| Splanchnic vascular resistance | No change | Decrease | No change | Decrease |
| Splanchnic oxygen consumption | No change | Slight increase | No change | Increase |
| Hepatic glucose output | No change | Increase | No change | Increase |
| Arterial lactic acid concentration | No change | Increase | Increase | Increase |
| Arterial glucose concentration | No change | Increase* | Increase* | Increase |

*From hypoglycemic levels.

## DISCUSSION

The present study shows clearly that hexamethonium in the dosage used does not potentiate insulin hypoglycemia in man. There are two possible explanations for this finding: either the dosage of hexamethonium used was not sufficient to prevent adrenaline release, or adrenaline was not solely responsible for the return of the blood glucose to normal following insulin hypoglycemia. Previous studies have shown that insulin hypoglycemia results in a rise in pulse pressure and in E.S.B.F., with a slight fall in splanchnic vascular resistance; in addition there is a rise in hepatic glucose output, splanchnic oxygen consumption, and peripheral lactic acid concentration[6] (Table IV). These effects are also produced by an infusion of adrenaline.[1] With hexamethonium and insulin, pulse pressure and E.S.B.F. fell, and splanchnic vascular resistance remained unchanged; similarly, both hepatic glucose output and

splanchnic oxygen consumption failed to show any significant rise. On the other hand, arterial lactic acid concentration rose, but this is said to be a less sensitive index of adrenaline release than the rise in blood glucose.[9]

It seems reasonable to conclude, therefore, that adrenaline release following insulin hypoglycemia is at least partially blocked by hexamethonium. If this is accepted, it is probable, as has been previously suggested,[6, 10] that some mechanism in addition to adrenaline may raise the arterial glucose concentration after insulin hypoglycemia.

There are two possible ways by which such a mechanism would be effective: increase in glucose output from the liver, or diminution in peripheral utilization of glucose. Hepatic glucose output may be altered by the hyperglycemic factor of the pancreas, glucagon,[10, 11] or by an inherent hepatic homeostatic regulating mechanism,[12] but neither of these seems likely here, since the response to hypoglycemia following hexamethonium and insulin is not accompanied by a rise in hepatic glucose output. Diminished utilization of glucose by muscles, kidneys, and intestines cannot be excluded, but this is difficult to assess in man.

The initial failure of the hepatic glucose output to fall to the same extent with hexamethonium and insulin, as with insulin alone, suggests that the blood glucose is being lowered by increased peripheral disposal of glucose. The failure of the hepatic glucose output to rise with the return of the blood glucose level toward normal, could thus be related to mobilization of this peripheral glucose, although the peripheral tissues are not usually considered a direct source of blood sugar.

Hepatic glucose output is calculated as the product of E.S.B.F. and the hepatic venous-arterial glucose difference. The significance of the present results depends on the validity of the techniques used for E.S.B.F. and hepatic venous glucose concentration. It is possible that the B.S.P. technique may not measure accurately the rapid fall in E.S.B.F. after hexamethonium. During the period following hypoglycemia, the circulation generally was not changing; however, the peripheral B.S.P. level and hepatic venous oxygen saturation remained constant, and the constant E.S.B.F. calculated under these conditions would therefore appear to be valid.[13] Bondy[14] has reported that there is no uniformity in the minute-to-minute hepatic vein glucose concentration in normal subjects, but analysis of samples drawn continuously from the hepatic vein over ten-minute periods has failed to demonstrate such fluctuations.[15] Further, the basal hepatic glucose output levels are strictly comparable to those reported previously from this laboratory[6-8] and elsewhere.[16] It seems reasonable to conclude that the hepatic glucose output calculations are valid.

At the present time, it is not possible to define further the mechanisms responsible for the restoration of the blood glucose concentration after the administration of hexamethonium and insulin. Changes in hepatic glucose output, measured by the hepatic vein catheterization technique, cannot alone account for the recovery.

SUMMARY

The hepatic vein catheterization technique has been used to study the circulatory and metabolic effects of soluble insulin (0.1 unit per kilogram intravenously) and hexamethonium bromide (1 mg. per kilogram intramuscularly) in twelve subjects. The hypoglycemic action of insulin is not potentiated by simultaneous hexamethonium administration. Adrenaline release in response to insulin hypoglycemia is partially blocked by hexamethonium. There is evidence that mechanisms other than adrenaline release may also be responsible for the restoration of arterial glucose concentration following insulin hypoglycemia.

We are indebted to Miss Frances Howard, B.Sc., for biochemical assistance and to the Lund Fund of the Diabetic Association (Great Britain) for an expenses grant.

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# AN ANALYSIS OF THE STRESS OF REALIMENTATION WITH CARBOHYDRATE OR PROTEIN FOLLOWING PROLONGED FASTING

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PREVIOUS studies[1] have shown that when fasting animals are realimented with *low maintenance* diets (60 cal./M²/hr./24 hr.) high in carbohydrate (cracker meal or boiled rice) or in protein (raw horse meat), they show prompt improvement and untoward reactions are usually absent provided that the first feeding is given slowly. When the same diets are fed to fasting animals at *luxus consumption* levels of intake (twice the low maintenance level), severe general, cardiovascular, and gastrointestinal reactions are common and are more intense with high carbohydrate than with isocaloric high protein diets. In a more recent study,[2] blood pressure and heart rate were studied when fasting dogs were realimented with the same diets. It was found that when the diets were fed at *low maintenance* levels the blood pressure and heart rate rose promptly but moderately above the fasting levels and there were no consistent or significant differences between the diets high in carbohydrate or in protein. When fed at *luxus consumption* levels, however, there was a definite difference between the high carbohydrate and the high protein diets. The high protein diet caused an elevation of blood pressure and pulse rate which was of the same order of magnitude as the low maintenance diets while an isocaloric diet high in carbohydrate caused a marked elevation of systolic blood pressure and heart rate which was very significantly greater than that caused by the high protein diet. The diastolic pressure was low or normal. The preliminary period of fasting was necessary to cause a maintained elevation of systolic pressure and heart rate by the high carbohydrate diet. The elevated pressure and heart rate were found to persist for as long as 108 days with no tendency to decrease but if an isocaloric high protein diet was substituted, the pressure fell promptly to the low level characteristic of the high protein diet.

A study of the eosinophil and total leukocyte counts suggested that pituitary-adrenal activity was in some way involved in the differences between the high carbohydrate and high protein diets. Because of certain well-known vagaries in the eosinophil count, it was considered essential to combine it with another indicator of pituitary-adrenal activity. In the dog[3] 17-ketosteroid excretion is apparently unreliable. Uropepsin excretion has been advocated for this purpose[4, 5] and was given a thorough trial but was found unsatisfactory for our purpose. At this time we became aware of the excellent work of Kramar and associates.[6-10] In a series of clear-cut experiments these investigators showed that the resistance of the capillary wall to external suction (capillary resistance-capillary fragility) is hormonally controlled, the resistance in both albino rats

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Financed by the Medical Research and Development Board, Office of the Surgeon General, Department of the Army under Contract No. DA-49-007-MD-337, and the National Heart Institute, National Institutes of Health, grant H-1014.

Received for publication, Jan. 21, 1954.

*Trainee under the Army Contract.

and dogs being increased by cortisone and decreased by adrenalectomy. DCA was without effect. STH antagonized the effect of exogenous cortisone and also caused a lowering of the resistance in normal rats.[10] All types of nonspecific stress which are known to activate the pituitary adrenal system caused an identical response of capillary resistance.[7] They also showed that there is an inverse relationship between capillary resistance and the circulating eosinophil count (rats and dogs).[8] This method seemed admirably suited to our needs and has been employed, with the eosinophil count, as an index of pituitary-adrenal activity.

The use of the level of circulating eosinophils and capillary resistance as indices of pituitary-adrenal activity does not imply that either is under the exclusive control of the pituitary-adrenal system. There is abundant evidence that other factors influence the eosinophil level and unpublished data from the laboratory of Kramar and of the present authors show the same for capillary resistance; therefore, in a strict scientific sense, their use for this purpose is based on an hypothesis. The response of these two factors, however, to adrenalectomy, exogenous cortisone, ACTH, nonspecific stresses of various kinds, as well as the opposite and antagonistic effects of cortisone and STH, appear to justify the concept that the level of pituitary-adrenal activity is at least a major factor in their control.

## METHODS

The original technique of Kramar and Simay-Kramar[6] was used, care being taken to recognize and avoid capillary spasm which, as they showed, may give erroneous, high values. Since they showed that emotional factors cause changes in capillary resistance,[9] all dogs were carefully trained before being used. The times of feeding and of determining the capillary resistance and eosinophil values were standardized and kept constant. In order to avoid the emotional stress caused by changing investigators,[11] each group of dogs was handled by the same person. Twelve dogs, seven males and five females, were studied.

Eosinophil and total leukocyte counts were done on free-flowing venous blood six days weekly using a standard Spencer bright line hemocytometer. The diluting fluid of Manners[12] was used for eosinophil counts.

Capillary resistance was determined on the skin of the anterior abdominal wall three times weekly except when rapid changes were occurring, when daily determinations were made.

After a control period of from twenty-five to fifty days, the animals were subjected to fasting (water ad libitum), until the capillary resistance had reached and stabilized at the fasting value.

In Part I of the study, five of the animals subjected to fasting were realimented with a luxus consumption high protein diet (raw horse meat) and four with the luxus consumption high carbohydrate diet (cracker meal or rice).* † In Part II, the five animals which had previously been realimented with the high protein diet were subjected to a second fast and realimented with the high carbohydrate diet; after the effects of the high carbohydrate diet had been determined, an isocaloric high protein diet (horse meat, raw beef, or purified casein) was substituted without an intervening fast. Three new dogs were added during this part.

## RESULTS

1. *Fasting.*—As shown in Figs. 1 and 2, the capillary resistance rose progressively during fasting and finally reached a maximal value of 69 to 70 cm. Hg in sixty-five and twenty-five days, respectively. Since 70 cm. Hg was the

*The rice and cracker meal were moistened and flavored with a solution of Difco beef extract and a tablespoon of brewer's yeast was added.

†One Multicebrin tablet (Eli Lilly & Company) was given daily to supply vitamins when on the high carbohydrate diets.

maximal vacuum obtainable, the true value may have been higher. Not all dogs reached this excessively high value, but, in dogs not previously subjected to severe dietary stresses, capillary resistance always rose very significantly above the control value during fasting.* The time required to reach the maximal fasting value varied from eleven to sixty-five days in the different dogs studied.

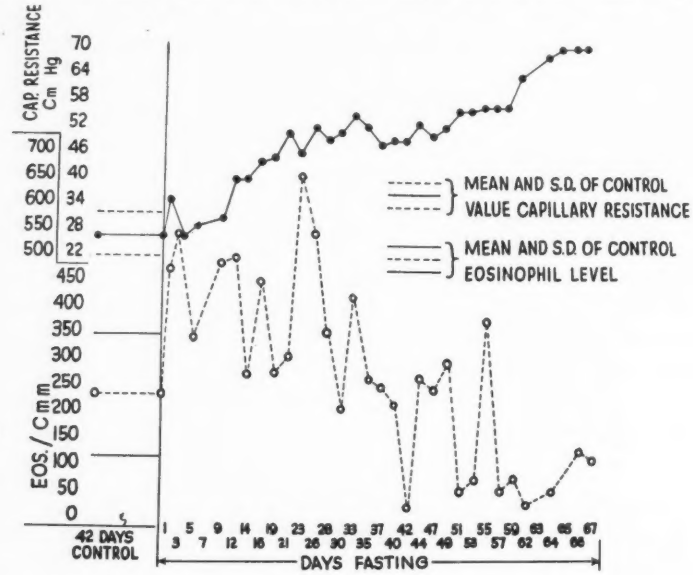


Fig. 1.

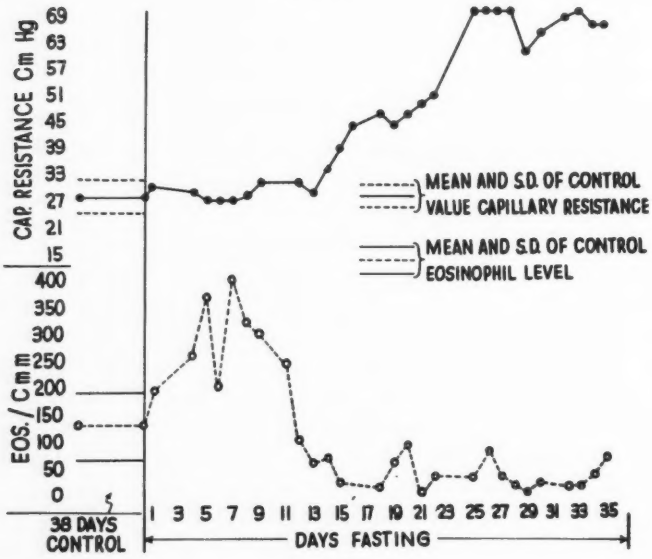


Fig. 2.

As the capillary resistance rose, the circulating eosinophils diminished and usually reached the minimal fasting values shortly before, or at the same time as, the capillary resistance reached its maximal value. During the early stages

*We have evidence that multiple prolonged fasts and realimentation with unbalanced diets may alter the response.

of the fast, when capillary resistance was hovering near the control level or rising slowly, the eosinophils characteristically showed marked fluctuation, which gradually diminished as the minimal fasting value was reached. If the control eosinophil value was very low, the change during fasting might not be statistically significant.

Depending on the rate of rise of capillary resistance and the fall in eosinophil level, the dogs could be divided into slow, medium, and fast reactors, and the type of reaction seemed to be related to the ability to successfully tolerate the fast.

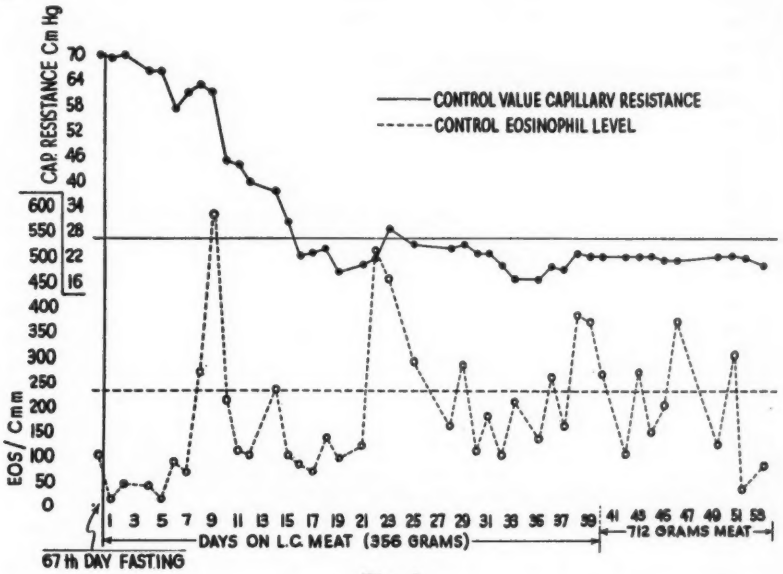


Fig. 3.

2. *Realimentation With High Protein Diets (Horse Meat).*—Fig. 3 shows the realimentation of the dog shown in Fig 1. After a short latent period, the capillary resistance began a rapid decrease and reached the control value on the sixteenth day. Approximately one week later the eosinophil count rose from the low fasting to the control value. Fig. 4 is the same dog shown in Fig. 2. Here also, the decrease of the capillary resistance was very rapid, the control value being reached in eight days. The eosinophils remained at the fasting level definitely longer than the capillary resistance (a common finding), but when recovery began, there was at first a marked "overshooting" followed by a return to the control value. In both dogs the decrease of the capillary resistance to the control value was much faster than the rise during fasting but it is interesting to note that the dog which had the slowest rise also had the slowest fall.

3. *Realimentation With High Carbohydrate Diets (Cracker Meal or Boiled White Rice).*—When fasting animals were realimented with diets high in carbohydrate, the response of the capillary resistance and the eosinophil count tended to be the reverse of the response to a diet high in protein, that is, the capillary resistance tended to remain high and the eosinophil count tended to remain low. In general there were three types of response of the capillary resistance:

A. To remain at the fasting level of 68 to 70 cm. Hg. In one dog realimented with rice, the capillary resistance remained at 70 cm. Hg for seventy-one days, with no tendency to diminish.

B. An initial decrease with a subsequent rise to or near the fasting level.

C. A decrease to, and stabilization at, a value below the fasting level, which was, however, significantly above the control value.

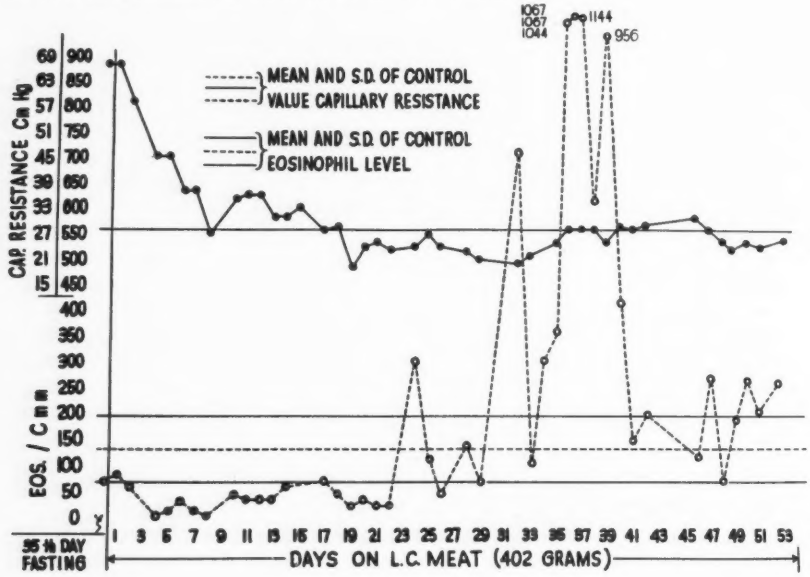


Fig. 4.

Following realimentation with diets high in carbohydrate, the eosinophil count usually rose somewhat above the fasting level but the mean values on the carbohydrate diet were usually significantly lower than the mean control level.

4. *Realimentation With Diets High in Carbohydrate, Followed Later by Substitution of an Isocaloric High Protein Diet.*—This type of experiment was performed on six animals. In one experiment the fasting animal was realimented with a luxus consumption diet of rice and the capillary resistance remained at the high level of 70 cm. for seventy-one days. An isocaloric purified casein diet was then substituted for the rice, and capillary resistance declined to normal in twenty-seven days.

Five other fasting animals were refed with luxus consumption diets of cracker meal and after the capillary resistance and eosinophil counts had become stabilized at the carbohydrate level, an isocaloric diet of ground raw beef or horse meat was substituted; in every experiment there was a rapid fall in capillary resistance and a rise in eosinophils. There was no apparent difference between beef or horse meat,* but both seemed to cause a more rapid fall in capillary resistance than casein.

Fig. 5 shows a complete experiment in which horse meat was substituted for cracker meal. In this experiment the high carbohydrate diet caused a decrease

*There are chemical differences between horse meat and beef but these did not alter the response

of capillary resistance below the fasting level, but the mean level on carbohydrate was very significantly above the control value. The eosinophil count remained at approximately the fasting value. When the high protein diet was substituted, the decrease in capillary resistance and the rise in eosinophils were

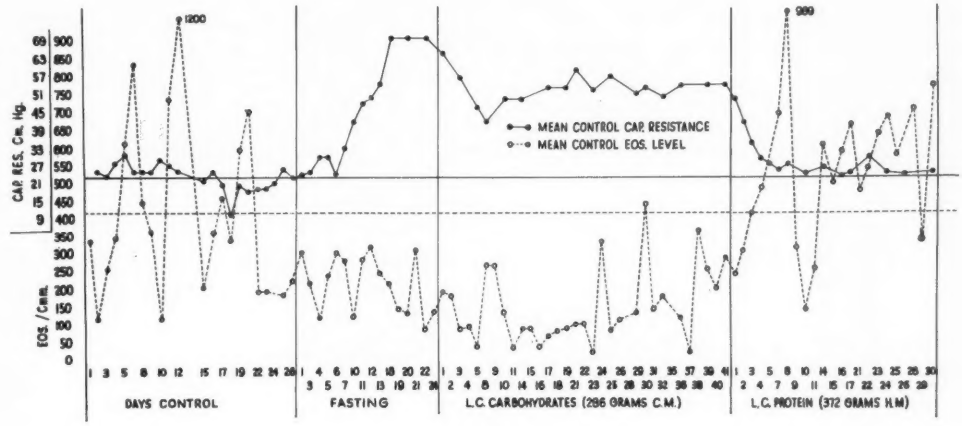


Fig. 5.

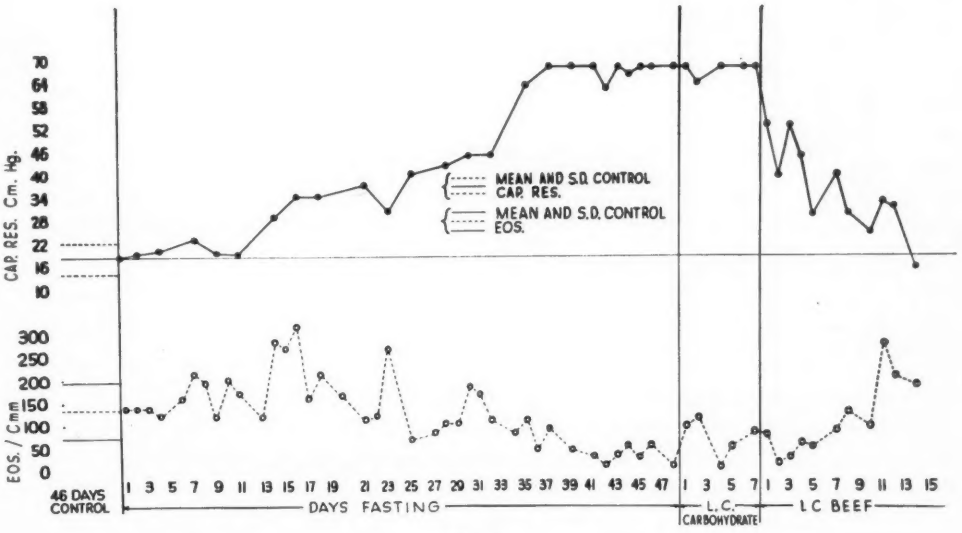


Fig. 6.

very rapid and striking. In several experiments (Fig. 3) after the capillary resistance and eosinophil count had returned to the control values on the luxus consumption meat diet, the amount of meat was doubled but in no instance did the capillary resistance rise or the eosinophil count decrease, thus showing that even this huge intake of meat did not constitute a stress in the sense that it activated the pituitary-adrenal system. Fig. 6 shows an experiment in which ground raw beef was used instead of horse meat.

5. *Exceptional Responses.*—A. One dog was realimented with the luxus consumption diet of cracker meal after fasting for thirty-nine days. For ten days

the capillary resistance and the eosinophil count remained at the fasting values and the animal appeared in excellent condition. It was found dead the next morning and autopsy revealed no obvious cause of death. It is possible that death was due to a severe cardiovascular reaction which may occur several days after realimentation with luxus consumption diets.[1]

B. After realimentation with the luxus consumption high carbohydrate diets, three dogs showed a spontaneous return of capillary resistance and eosinophil counts to the control values. These spontaneous recoveries were slow and it required fifty, fifty-two, and sixty days, respectively, for the capillary resistance to reach control values. As shown in Fig. 7, the administration of corti-

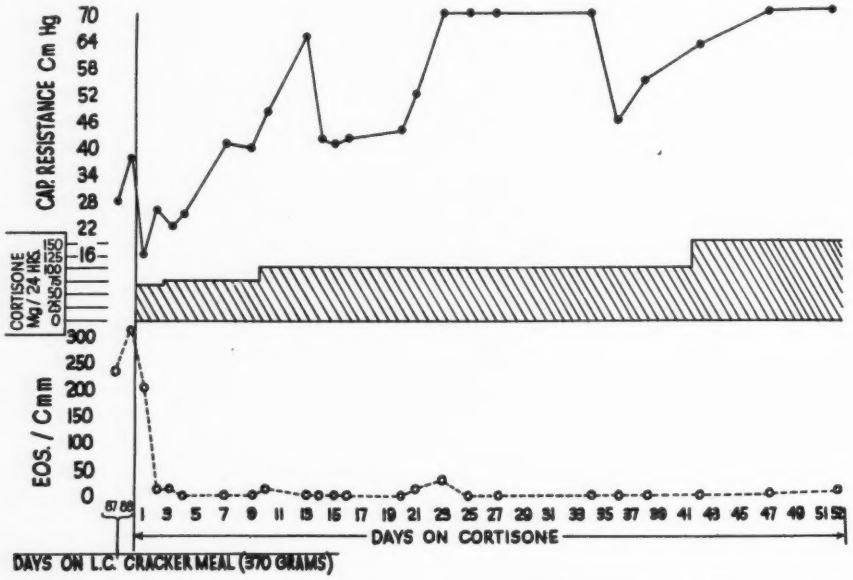


Fig. 7.—The effect of cortisone on the capillary resistance and eosinophil count in a dog which had shown spontaneous recovery while on the high carbohydrate diet. The carbohydrate diet was continued during the period of cortisone administration.

sone, after spontaneous recovery, caused the capillary resistance and eosinophil counts to return to the fasting values, thus showing that the target organs were capable of response. The slow rise of the capillary resistance in spite of the large doses of cortisone is probably due to the fact that cortisone utilization was great because of the very high carbohydrate diet. This experiment also gives some approximation of the amount of endogenous gluco-corticoids which must be liberated from the adrenal cortex to adequately maintain high carbohydrate utilization and also keep the capillary resistance at the high values often observed.

One dog which showed a spontaneous return to control values was given 40 Armour Units of ACTH in gelatin subcutaneously, which caused a significant elevation of capillary resistance and a decrease in the eosinophil count. This suggested depletion of the ACTH content of the pituitary due to the long period of fasting and subsistence on an unbalanced diet. A second and third injection gave very little response which suggests that the adrenal cortex was also par-

tially depleted and incapable of rapidly replenishing its hormone content because of the inadequate diet.

Daily blood pressure determinations were being made on two of the dogs that showed a spontaneous return to normal. The decline of the systolic blood pressure, heart rate, and capillary resistance to normal, and the rise of the eosinophil count were almost simultaneous. The blood pressure rose to high levels on cortisone administration.

C. Several experiments were performed in which the luxus consumption high carbohydrate diet was substituted for the kennel diet (Nutrena) without a preliminary fast. In these experiments the eosinophil count slowly diminished but there was no rise in capillary resistance. This is reminiscent of the behavior of the blood pressure under similar conditions[2] and suggests that the high carbohydrate diet alone causes only a minimal increase in the activity of the pituitary-adrenal system, the preliminary fast being necessary for the maximal increase which can then be maintained by the carbohydrate diet.

## DISCUSSION

The present studies show that luxus consumption diets high in carbohydrate tend to maintain the stress engendered by prolonged fasting, while isocaloric diets high in protein tend to abolish rapidly both the stress engendered by fasting, per se, as well as that maintained by high carbohydrate diets; thus high protein diets have effects which are antagonistic as well as opposite to the effects of high carbohydrate diets. It is important to emphasize that previous studies[2] showed the same opposing and antagonistic effects of protein and carbohydrate on systolic blood pressure and heart rate when fed to animals after prolonged fasting. The severe untoward reactions occurring after realimentation of the fasting animal were also maximal with carbohydrate and minimal with protein.[1] It is important to note that these opposing effects of protein and carbohydrate were seen only when these foodstuffs were fed to the fasting animals in luxus consumption, not in low maintenance levels of intake, thus the stress of overeating (quantity) seems necessary to bring out the qualitative differences between protein and carbohydrate. Fasting is an alarming stimulus and sensitizes to other alarming stimuli.[13] This was confirmed by showing that the characteristic effects of carbohydrate on systolic blood pressure and heart rate were minimal and temporary when luxus consumption high carbohydrate diets were substituted for the usual kennel diet without a preliminary fast.[2]

There was a close relationship between the elevation of the systolic blood pressure, heart rate, and capillary resistance, and the lowering of the eosinophil count on the luxus consumption high carbohydrate diets, since all tended to return to normal values when spontaneous recovery occurred. The nature of the response to ACTH and cortisone after spontaneous recovery suggests the possibility that spontaneous recoveries may have been due, at least in part, to depletion of the hormones of the pituitary-adrenal system. If subsidence of the untoward manifestations of realimentation with luxus consumption high carbohydrate diets was associated with hypofunction of the pituitary-adrenal system, then it seems logical to conclude that their inception may have been associated with hyperfunction.

SUMMARY

During prolonged fasting there was an elevation of capillary resistance and a decrease of circulating eosinophils, an effect which can be produced in non-fasting animals by cortisone. Realimentation with luxus consumption diets high in carbohydrate (rice or cracker meal) tended to maintain the fasting levels, while realimentation with isocaloric diets high in protein (casein, beef, or horse meat) caused a very rapid return to normal values either when fed initially to the fasting animal or after the high carbohydrate diet.

The carbohydrate effect may persist unaltered for as long as seventy-one days; on the other hand, three dogs showed a slow spontaneous return to normal while on the carbohydrate diet, which required from fifty to sixty days. After spontaneous recovery, cortisone and ACTH caused, respectively, complete and partial return of the capillary resistance and eosinophil levels to the high carbohydrate values. Systolic blood pressure and pulse rate showed similar behavior during spontaneous recovery and administration of cortisone. This suggests that spontaneous recovery may be associated with depletion of the pituitary-adrenal hormones.

On the basis of these and previously reported studies, it is suggested that the stress engendered in fasting animals by realimentation with luxus consumption diets high in carbohydrate is associated with hyperactivity of the pituitary-adrenal system whch is counteracted by isocaloric high protein diets.

Without the expert and enthusiastic technical assistance of Mr. P. G. Green, many phases of this work would not have been possible, and we wish to express to him our grateful thanks.

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# NEWER STANDARDS IN HEMOGLOBINOMETRY

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DETERMINATION of oxygen-carrying capacity or of the total iron content in a sample of blood are the two standard methods used in the calibration of photometric instruments for the measurement of oxyhemoglobin.[1, 2] In order to obviate those elaborate procedures which often are not available, Drabkin proposed an ingenious method, employing, in essence, a single, easily prepared solution of cupric ammonium sulfate.[3-5] The method is based on the empirical finding that the ratio of optical densities of two independent color standards is reasonably constant for all photoelectric photometers of the same type. Thus, the ratio (hereafter designated as DR ratio) of the optical density of a standard solution of oxyhemoglobin (blood containing 15 Gm. of hemoglobin per 100 ml., in suitable dilution) to the density of a standard cupric ammonium sulfate solution was found to be constant among six Klett-Summerson photometers.[4, 5] It follows that all the owner of another Klett photometer has to do to calibrate his instrument is to prepare gravimetrically the standard copper solution, determine its optical density ($D_{Cu}$) and multiply this value by the DR ratio characteristic of his make of instrument, viz., 0.984.[4] The result is the optical density of the standard blood which permits calculation of the calibration factor (K)[6] for the instrument:

$$K = \frac{\text{Concentration of standard}}{\text{Density of standard}} = \frac{15}{DR \times D_{Cu}}.$$

The hemoglobin concentration of any other blood sample (Hb) can then be readily determined from its optical density reading ($D_{HbO_2}$) by means of the usual equation:

$$Hb = D_{HbO_2} \times K.$$

Attractive as this method of calibration seemed to be, the relatively small number of photometers tested† made it desirable to obtain additional confirmatory evidence before acceptance.

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Received for publication, Jan. 27, 1954.

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†Seven Klett-Summerson photometers, two Evelyn photometers, two General Electric recording spectrophotometers, two Beckman photoelectric quartz spectrophotometers, and three Bausch and Lomb spectrophotometers.[5] (Note: Trade names are used throughout as a means of identifying the products under discussion and do not represent endorsement by the Public Health Service.)

The availability of limited amounts of purified reduced hemoglobin[7] (hereafter abbreviated RHb*) provided another approach to the simplified measurement of oxyhemoglobin. If this reduced hemoglobin solution, with its exact concentration supplied by the manufacturer, could be converted to, and measured as, oxyhemoglobin in the manner routinely applied to blood, it would constitute a nearly ideal standard.

In the investigation hereinafter reported, an attempt was made to determine (a) the reproducibility of the value of the DR ratio among instruments of three types, i.e., ten Coleman Junior spectrophotometers (Model 6A), three Coleman Universal spectrophotometers (Model 14), and six Klett-Summerson filter photometers; and (b) the reliability of RHb as a standard in oxyhemoglobinometry.

## MATERIALS AND METHODS

A sample of fresh oxalated venous blood (designated B), after thorough mixing, was divided into five portions, one to be analyzed immediately, the other four portions to be stored at 4° C. for analysis on the following four days. A sample of RHb was withdrawn on each of those 5 days from a single, air-evacuated, rubber-stoppered vial, which was kept at 4° C., and treated in the same manner as the blood. The Wong method for total iron concentration[1, 8] was carried out daily for five days on triplicate tungstate filtrates of duplicate samples of both blood B and RHb. (However, results of analyses performed the fifth day on blood B were discarded because of partial clotting of the blood.) Calculation of hemoglobin concentrations was based on an iron content of 0.338 per cent of the hemoglobin molecule.[9] Optical density readings for oxyhemoglobin were taken each day on duplicate specimens of both blood B and the RHb material, each diluted 1:251 with 0.04 per cent ammonium hydroxide† by means of volumetric flasks and a single accurately calibrated 1 ml. pipette. The readings were taken each day in the course of about two hours on all 19 instruments mentioned above at a wave length of 540 mμ (or green filter No. 54), using the diluting fluid as a blank. Simultaneous duplicate density readings were made on a 0.012 M cupric sulfate standard,[3-5] which was made up with anhydrous $CuSO_4$, dried at 300° C. (3.8328 Gm. were dissolved in 235.2 ml. of concentrated ammonium hydroxide and sufficient distilled water was added to bring the total volume to 2,000 ml.).

## RESULTS

Since calculation of the DR ratios and other parameters involved hemoglobin values based on the measurement of total iron content in a number of tungstate filtrates made from aliquots of blood B and reduced hemoglobin, analyses of variance of these values were made.[10] These analyses indicated that the differences observed from day to day were not significant and thus permitted the averages, i.e., 11.71 Gm. per 100 ml. for B and 6.96 for RHb, to represent the respective hemoglobin concentrations.

The DR ratio was calculated by the formula, $DR = \frac{D_{BHbO_2}}{D_{Cu}} \times \frac{15}{11.71}$, with $D_{BHbO_2}$ representing the optical density of the 1:251 dilution of blood B. It is apparent from Table I that the variability of the ratio (as expressed by the coefficient of variation[10]) for the same instrument on different days was small.

*Supplied through the courtesy of Dr. W. P. Boger of Sharpe and Dohme, Inc., Philadelphia, Pa. The hemoglobin concentration marked on the label was 7.05 gm. per 100 ml.

†In accordance with the recommendations of the American Society of Clinical Pathologists' Symposium on Hemoglobinometry.[1]

This variability was also small when determined among the Klett photometers (coefficient of variation = 0.6 per cent) over the four-day test period; however, it was considerable among the Coleman Junior and Universal spectrophotometers (C.V. = 3.4 and 4.2 per cent, respectively). Thus, if conclusions are permitted on the basis of the small number of instruments tested, one may state that 95 per cent of DR ratio values furnished by Klett instruments may be expected to fall within ± 1.2 per cent of the true value, a percentage which is well within the range of ± 5 per cent usually accepted in clinical hemoglobinometry. This does not seem to be true of the spectrophotometers in which the quoted figures exceed these limits.

TABLE I. DR RATIOS AS DETERMINED ON THREE TYPES OF PHOTOMETERS BY DUPLICATE READINGS OF $D_{BHbO_2}$ AND $D_{Cu}$ ON EACH OF FOUR CONSECUTIVE DAYS

| PHOTOMETER NO. | DR RATIOS | | 2 C. V.* (%) | PHOTOMETER NO. | DR RATIOS | | 2 C. V.* (%) |
|---|---|---|---|---|---|---|---|
| | RANGE | MEAN | | | RANGE | MEAN | |
| J1 | 0.950-0.974 | 0.960 | 1.71 | U1 | 0.990-1.021 | 1.005 | 2.12 |
| J2 | 0.970-0.991 | 0.981 | 1.58 | U2 | 0.914-0.947 | 0.932 | 2.83 |
| J3 | 0.979-1.004 | 0.992 | 1.64 | U3 | 0.978-1.008 | 0.996 | 1.82 |
| J4 | 0.957-0.980 | 0.973 | 1.61 | All U Photometers | 0.914-1.021 | 0.978 | 8.30† |
| J5 | 0.953-0.969 | 0.957 | 2.01 | K1 | 0.994-1.030 | 1.017 | 2.26 |
| J6 | 0.922-0.948 | 0.936 | 1.96 | K2 | 0.982-1.032 | 1.007 | 3.36 |
| J7 | 0.939-0.985 | 0.963 | 3.87 | K3 | 0.985-1.038 | 1.014 | 4.45 |
| J8 | 0.932-0.956 | 0.948 | 1.90 | K4 | 0.992-1.019 | 1.005 | 1.72 |
| J9 | 0.963-0.990 | 0.976 | 2.14 | K5 | 1.004-1.019 | 1.010 | 1.39 |
| J10 | 0.870-0.888 | 0.875 | 1.39 | K6 | 1.008-1.031 | 1.020 | 1.82 |
| All J Photometers | 0.870-1.004 | 0.957 | 6.84† | All K Photometers | 0.982-1.038 | 1.012 | 1.24† |

$$\text{DR Ratio} = \frac{D_B HbO_2}{D_{Cu}} \times \frac{15}{11.71}$$ (See text for explanation of symbols.)

$$\text{C.V.} = \text{Coefficient of Variation} = \frac{\text{Standard Deviation}}{\text{Mean}} \times 100.$$

*2 C.V. of all DR ratios obtained over 4 days per photometer.
†2 C.V. of all DR ratios obtained over 4 days on all photometers of the same type.
J denotes Coleman Junior spectrophotometers.
U denotes Coleman Universal spectrophotometers.
K denotes Klett-Summerson filter photometers.

For evaluation of the reliability of RHb as a standard, it was obviously necessary to determine whether all or only part of this substance could be converted to oxyhemoglobin by the routine 251-fold dilution with ammonium hydroxide. In other words, was the optical density of the diluted RHb identical with that of a similarly treated blood sample having the same total iron (hemoglobin) concentration? If the density of $RHbO_2$ was different, this material could still be visualized as a suitable standard, provided that the relationship between expected and observed densities was constant, so that, perhaps, a correction factor might be worked out, as had been recommended with the use of crystalline hemin as a standard.[11, 12]

Instead of searching for a blood sample which had an iron concentration exactly equal to that of RHb, it was reasoned that the answer to this question

could more readily be secured by calculating, for each photometer, the product (RHb′), density of the RHb solution ($D_{RHbO_2}$) times the calibration factor* derived from blood B:

$$RHb' = D_{RHbO_2} \times K_B, \text{ since, ideally, } \frac{RHb}{D_{RHbO_2}} = \frac{BHb}{D_{BHbO_2}} = K_B, \text{ and}$$

RHb′ = actual hemoglobin content of RHb. If, however, these latter values were found to differ consistently, then this difference reflected an "inert" fraction of the reduced hemoglobin material that could not be converted to oxyhemoglobin; RHb′ could then be regarded as *functional hemoglobin concentration* or as *hemoglobin equivalent*, representing the hemoglobin concentration of a hypothetical blood sample that would yield the same optical density as RHb.

TABLE II. ANALYSES OF VARIANCE OF CALIBRATION FACTOR ($K_B$) VALUES AS DETERMINED BY DAILY READING OF $D_{BHbO_2}$ FOR FOUR DAYS

| TYPE OF INSTRUMENTS | SOURCE OF VARIATION | DEGREES OF FREEDOM | MEAN SQUARES | F RATIOS |
|---|---|---|---|---|
| Coleman Junior spectrophotometers | Days | 3 | 0.665 | 13.2† |
| | Photometers | 9 | 172.803 | 3,200.0† |
| | Interaction D × P | 27 | 0.067 | 1.3$^{NS}$ |
| | Duplicates | 36 | 0.052 | -- |
| Klett-Summerson filter photometers | Days | 3 | 7.533 | 3.6* |
| | Photometers | 5 | 35.104 | 16.9† |
| | Interaction D × P | 15 | 2.080 | 6.4† |
| | Duplicates | 24 | 0.325 | -- |

$K_B = \frac{11.71}{D_{BHbO2}}$ (See text for explanation of symbols.)

*Significant at the 5 per cent probability level.

†Significant at the 1 per cent probability level.

NS—Not Significant at the 5 per cent probability level.

$K_B$ values obtained from Coleman Universal spectrophotometers were not subjected to this analysis because of the small number (3) of these instruments available.

The coefficients of variation, as assessed from differences in duplicates, were 0.81 per cent and 0.77 per cent for the Coleman Junior and Klett-Summerson instruments, respectively.

The mean hemoglobin equivalents for the three groups of instruments, Coleman Universal and Junior spectrophotometers and Klett filter photometers, were 6.52, 6.69, and 6.68 Gm. per 100 ml., respectively, (Table III) with an over-all mean of 6.63. Since these values, as well as, with one exception, all individual daily values from which these means were computed, fell distinctly short of 6.96 Gm. per 100 ml., (i.e., the hemoglobin concentration of RHb; range of thirty replicate iron analyses: 6.88-7.04), the inference is that a small portion of this hemoglobin (3.9 to 6.3 per cent) could not be converted to oxyhemoglobin by dilution with ammonium hydroxide and thus altered the photometric reading.

The variation of hemoglobin equivalents per instrument from day to day, as well as from instrument to instrument of each type tested, was quite small.

*Since analysis of variance of $K_B$ values, as seen in Table II, indicated a significant difference from day to day on the same photometer, a common experience with these instruments, this factor was determined on each of the four test days for calculation of corresponding hemoglobin equivalents. Actually, this variation, though statistically significant, was quite small. For both types of Coleman spectrophotometers, all $K_B$ values were within ± 2 per cent of the mean per instrument. On the Klett-Summerson filter photometers, all but one value fell within ± 3 per cent of the mean $K_B$ per instrument. The significant interaction between Klett photometers and days was caused by the failure of *one* instrument ($K_6$) to reproduce the day-to-day fluctuations shown by the whole group of Klett instruments.

Hence, it appears that the mean hemoglobin equivalent for each photometer type may be considered the effective or functional hemoglobin concentration for use in calibration and should take the place of the RHb value obtained by chemical analysis. A high degree of constancy of the hemoglobin equivalents was demonstrated by the absence of trend in the day-to-day fluctuation of values and by the observation that they were not materially affected by storage of RHb at 4° C. for one year: results obtained on one Coleman Universal, one Junior, and one Klett photometer, each recalibrated in the manner outlined previously by means of a fresh blood sample, were now 6.54, 6.57, and 6.69 Gm. per 100 ml., respectively, giving an over-all mean of 6.60, as against the mean RHb′ of 6.63 of one year previously. This finding indicates a remarkable stability of the RHb material.

TABLE III. HEMOGLOBIN EQUIVALENTS OF PURIFIED REDUCED HEMOGLOBIN (RHb) AS DETERMINED ON THREE TYPES OF PHOTOMETERS BY DUPLICATE READINGS OF $D_{RHbO_2}$ ON EACH OF FOUR CONSECUTIVE DAYS

| PHOTOMETER NO. | Hb. EQUIVALENTS RANGE | Hb. EQUIVALENTS MEAN | 2 C.V.* (%) | PHOTOMETER NO. | Hb. EQUIVALENTS RANGE | Hb. EQUIVALENTS MEAN | 2 C.V.* (%) |
|---|---|---|---|---|---|---|---|
| J1 | 6.77-6.90 | 6.83 | 1.46 | U1 | 6.39-6.57 | 6.51 | 2.11 |
| J2 | 6.57-6.73 | 6.65 | 1.62 | U2 | 6.44-6.65 | 6.54 | 2.72 |
| J3 | 6.49-6.72 | 6.63 | 2.38 | U3 | 6.10-6.73 | 6.52 | 6.43 |
| J4 | 6.52-6.73 | 6.66 | 2.12 | All U Photometers | 6.10-6.73 | 6.52 | 0.49† |
| J5 | 6.59-6.73 | 6.65 | 1.46 | K1 | 6.64-6.80 | 6.74 | 2.43 |
| J6 | 6.60-6.77 | 6.68 | 2.00 | K2 | 6.69-6.95 | 6.80 | 3.12 |
| J7 | 6.48-6.79 | 6.63 | 3.14 | K3 | 6.49-7.00 | 6.76 | 4.25 |
| J8 | 6.46-6.71 | 6.60 | 2.68 | K4 | 6.32-6.78 | 6.64 | 4.85 |
| J9 | 6.48-6.65 | 6.58 | 1.75 | K5 | 6.38-6.80 | 6.56 | 3.93 |
| J10 | 6.89-7.01 | 6.95 | 1.47 | K6 | 6.19-6.84 | 6.57 | 6.25 |
| All J Photometers | 6.46-7.01 | 6.69 | 3.37† | All K Photometers | 6.19-7.00 | 6.68 | 2.96† |

Hemoglobin Equivalent $= K_B \times D_{RHbO_2}$ (See text for explanation of symbols.)

C.V. = Coefficient of variation $= \frac{\text{Standard Deviation}}{\text{Mean}} \times 100.$

*2 C.V. of all hemoglobin equivalents obtained over four days per photometer.

†2 C.V. of all hemoglobin equivalents obtained over four days on all photometers of the same type.

J, U, K, see Table I.

## DISCUSSION

In two publications, two years apart, Drabkin quotes two different figures for the DR ratio, as derived from six Klett filter photometers, 0.984[4] and 0.963.[5] These values are about 2.8 and 4.9 per cent, respectively, lower than the mean DR ratio of 1.012 obtained from six Klett instruments in this study. The reasons for these discrepancies are not immediately apparent but may, at least in part, be sought in differences in techniques of preparing the copper standard and of measuring the hemoglobin content of the blood serving as standard. Thus, Drabkin used $CuSO_4.5H_2O$, while anhydrous copper sulfate appeared to the authors more desirable for gravimetric measurement. The hemoglobin standard chosen by Drabkin was blood of precisely 15 per cent concentration (though it is not clear how this concentration was determined or prepared),[4, 5] whereas the

15 per cent standard used in this study was a hypothetical blood whose density was computed from the hemoglobin concentration and optical density of a random blood sample. Moreover, the reagent used in making up the working dilutions of hemoglobin required for photometric reading was 0.1 per cent $NaHCO_3$ in Drabkin's case, and 0.04 per cent $NH_4OH$ in the present work. In view of these technical differences, the disparity between even Drabkin's lower value[5] and the DR ratio of the authors is not at all surprising. On the contrary, it is rather gratifying to learn that the differences in hemoglobin results, whether calculated on the basis of Drabkin's or our DR ratios, will be no greater than 5 per cent, a margin of error which is satisfactory in clinical work.

The sensitivity of individual photocells and transmittance of light filters, although they may vary somewhat among instruments of the same type and affect optical density readings of a single solution, do not greatly influence the ratios of densities of two solutions.[4, 5] Both Drabkin's and the authors' determinations of DR ratios among six separate Klett instruments agreed within about 1 per cent (Table I). It seems to be a safe conclusion that use of the DR ratio affords not only a simple but reliable method of calibrating Klett photometers for oxyhemoglobinometry. This conclusion may well be extended to other photometers provided with filters which transmit relatively broad bands of the spectrum.[4, 5]

In obvious contrast to these findings was the marked variation in DR ratios encountered among different spectrophotometers of the same type. The narrower spectral span of these instruments appears to make them unsuitable for calibration by the DR ratio method.

This limitation does not apply to the use of purified reduced hemoglobin as a standard. Results of hemoglobin equivalents among the three types of instruments tested were within so small a range as to make this material appear both useful and reliable for the calibration of any of these instruments, provided that the hemoglobin equivalent and not the actual hemoglobin content is substituted for RHb in calculating the calibration factor, $K = \frac{RHb}{D_{RHbO_2}}$. Once this factor is established for a photometer, of either the spectral or filter type, measurement of hemoglobin of an unknown blood sample will require only its optical density reading: $Hb = D_{HbO_2} \times K$. At the moment it appears that the hemoglobin equivalent will have to be measured (either by the manufacturer or some reliable laboratory) for each vial, or at least each batch of vials, of RHb material. In view of the small differences among hemoglobin equivalents for the three types of instruments used, one may anticipate that results obtained with other photometers having similar wave length spans will correspond closely in magnitude. Results may be appreciably different, however, on an instrument such as the Beckman DU spectrophotometer, which transmits, for each wave length setting, a spectral band far narrower than either of the two Coleman models studied. With this limitation in mind, it may be permissible to speak of an over-all average hemoglobin equivalent (viz., 6.63 Gm. per 100 ml. for the authors'

material) and to utilize this value in the routine calibration of photometers. This practice seems to be the more acceptable since the difference encountered between actual hemoglobin concentration and mean hemoglobin equivalent was less than 5 per cent of the former (RHb–mean RHb′ = 6.96 – 6.63 = 0.33 Gm. per 100 ml. = 4.7 per cent of RHb).

Once determined, the hemoglobin equivalent may be expected to be stable for at least one year if the material is stored under proper refrigeration conditions and kept from exposure to oxygen in order to minimize conversion to methemoglobin or other hemoglobin derivatives.[9]

## SUMMARY AND CONCLUSIONS

The validity of a gravimetrically prepared cupric ammonium sulfate solution and of a commercially supplied stable solution of reduced hemoglobin as color standards in oxyhemoglobinometry has been investigated. For evaluation of the copper standard, repeated optical density readings of both a blood specimen of known hemoglobin content and the standard were secured on three types of photometers (nineteen instruments), and the respective ratios of densities (DR ratios) computed in accordance with Drabkin's principle. The reduced hemoglobin standard was studied by comparing its actual hemoglobin content (as obtained by iron analysis) with hemoglobin values derived from replicate photometric measurements on those same instruments, calibrated by means of the known blood.

The DR ratios furnished by six Klett-Summerson filter photometers fell within ± 1.2 per cent of the mean DR value, while the corresponding ranges for ten Coleman Junior and three Coleman Universal spectrophotometers were ± 6.8 and ± 8.3 per cent, respectively. The better reproducibility of DR ratios in the case of Klett photometers is attributed to the greater width of the spectral band which is inherent in filter photometers and apparently is a prerequisite for validity of the DR ratio principle. Consequently, use of the copper standard can be recommended for filter photometers but not for spectrophotometers.

The variation of photometric hemoglobin values of the reduced hemoglobin material (hemoglobin equivalents) was about ± 4 per cent among the Coleman Junior spectrophotometers and ± 3 per cent among the Klett photometers. Hence, this standard appears to be equally applicable to both types of instruments. Hemoglobin equivalents, however, were from 3.9 to 6.3 per cent, on the average, lower than the actual hemoglobin content, indicating a small fraction of hemoglobin that failed to be converted to oxyhemoglobin by the diluent (ammonium hydroxide) employed. Therefore, the hemoglobin equivalent rather than the actual hemoglobin concentration of the standard is required for reliable photometric measurement of oxyhemoglobin in blood by this means. The reliability of the method is enhanced by a considerable stability of the material if kept under proper refrigeration and anaerobic conditions.

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# THE EFFECT OF VITAMIN $B_{12}$ AND FOLIC ACID ON NUCLEIC ACID COMPOSITION OF THE BONE MARROW OF PATIENTS WITH MEGALOBLASTIC ANEMIA

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## INTRODUCTION

VITAMIN $B_{12}$, folic acid, and ascorbic acid have induced partial or complete hematologic remissions in persons with various types of megaloblastic anemia. The impressive changes in the blood, bone marrow, and clinical well-being caused by these vitamins must be mediated through profound changes in cellular chemistry. Biochemical, microbiologic, and cytologic evidence implicates these vitamins in the formation and metabolism of the nucleic acids or of their most important constituents, the purine and pyrimidine bases. Therefore, we have investigated the changes that occur in the nucleic acid composition of the bone marrow of patients with megaloblastic anemia before, during, and after therapy with these vitamins. Before treatment, the concentrations of RNA and uracil are high compared with DNA and thymine. After treatment, normal relationships are restored.

## MATERIALS AND METHODS

Twenty-nine subjects were studied. Seventeen were normal hematologically and were used as controls. One had sprue and eleven had pernicious anemia. Treatment was given in one or more of the following ways: (1) ascorbic acid, orally, 1,000 mg. daily; (2) vitamin $B_{12}$ intramuscularly in suboptimal doses of 0.25 $\gamma$ daily for ten days or in optimal doses of 5 to 30 $\gamma$ daily; (3) folic acid intramuscularly in suboptimal doses of 0.5 mg. daily for ten days or in optimal doses of 10 mg. daily for ten days; (4) folinic acid intramuscularly in doses of 1 mg. daily for ten days.

Hematologic studies including erythrocyte and reticulocyte counts and hematocrit and hemoglobin determinations were made at regular intervals on all patients. Bone marrow was obtained by aspiration from the sternum or iliac crest marrow cavities before, during, and after therapy. Cover slip films were prepared, stained with Wright-Giemsa stain, and differential nucleated cell counts performed. The remainder of the marrow was put into a tube containing heparin and iced immediately to inhibit activity of the nucleases. The sample was centrifuged in Wintrobe tubes in the cold and the buffy coat recovered, mixed with a small amount of saline solution or $H_2O$ and frozen if it could not be processed at that time.

Precipitation of the nucleoproteins and removal of the acid-soluble phosphorus with cold trichloracetic acid and the lipids with hot alcohol-ether were accomplished in the usual manner after careful homogenization in a glass homogenizer. The residue was dried in a vacuum desiccator and weighed.

One portion, about 4 to 5 mg., was extracted with 5 per cent TCA by heating to 90° C. to obtain the nucleic acids. RNA and DNA were determined by the orcinol and diphenylamine methods of Mejbaum and Dische, respectively, as described by Schneider.[1]

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This work has been supported by grants from Lederle Laboratories and from the National Vitamin Foundation.

Presented in part before the 26th Annual Meeting of the Central Society for Clinical Research, Oct. 30-31, 1953.

Received for publication, Feb. 1, 1954.

The other portion was used for the determination of the purine and pyrimidine bases by paper chromatography according to the method of Marshak and Vogel[2] modified to adapt it to bone marrow analysis as follows: A quantity of glucose approximately equal in weight to the dried material was added before hydrolysis with perchloric acid. This decreased the amount of color and fluorescing material in the hydrolysate supernatant.* After heating in a boiling $H_2O$ bath for an hour with 70 per cent $HClO_4$, the hydrolysate was diluted with a minimal quantity of $H_2O$. The acid was neutralized and the perchlorate ion precipitated by the addition of 12N KOH, approximately equal in volume to the amount of $HClO_4$ used. The mixture was centrifuged to separate out the charred sugars and precipitated $KClO_4$. The supernatant varied with different specimens from almost colorless to light brown.

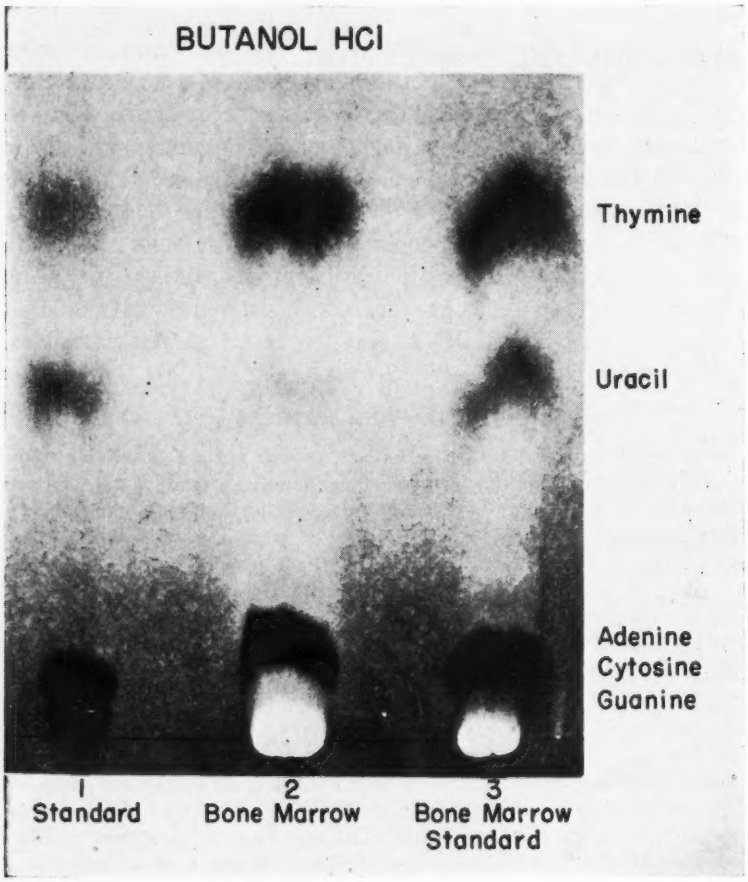


Fig. 1.—A paper chromatogram, photographed in ultraviolet light, demonstrating the separation of uracil and thymine by the acid-butanol system.

This material was applied to the paper for chromatography in amounts from 8 to 40 $\mu$l by means of an 8 $\mu$l ultramicro pipette, using warm air to dry the area between applications in order to maintain as fine a line as possible. Five lanes were run on the same paper, one for a blank, one for a standard, two for bone marrow in duplicate, and one for bone marrow with standard also applied.

An ascending chromatogram was run using a butanol-1N HCl (7:1) system for the separation of thymine and uracil[4] (Fig. 1) or butanol-0.1 N ammonia (7:1) system for the

*Schneider[1] and Cohen[3] found that a fluorescent, colored compound was formed when tryptophane and desoxyribose were heated with $HClO_4$. Glucose does not give fluorescence when treated in this way so it was added to compete with and replace desoxyribose in the formation of a tryptophane-sugar condensation product.

separation of thymine, cytosine, guanine, and a mixture of adenine and uracil[4] (Fig. 2). After completion of the run (about eighteen hours) the paper was air dried and examined under ultraviolet light (SL Mineralight with Corning 2537 short-wave filter). Because of the small amount of material, the standard lanes were used as guides in locating the zones containing the desired bases. These zones were cut out, eluted with 2 ml. of 0.1 N HCl and the eluate examined in a Beckman DU spectrophotometer. The quantity of base was calculated from the optical density as described by Vischer and Chargaff.[4] Uracil cannot be separated from adenine in the alkaline butanol system so the mixture was calculated as adenine. Since both bases have maximum absorption at the same wave length and the constant does not differ greatly, no large error was introduced.

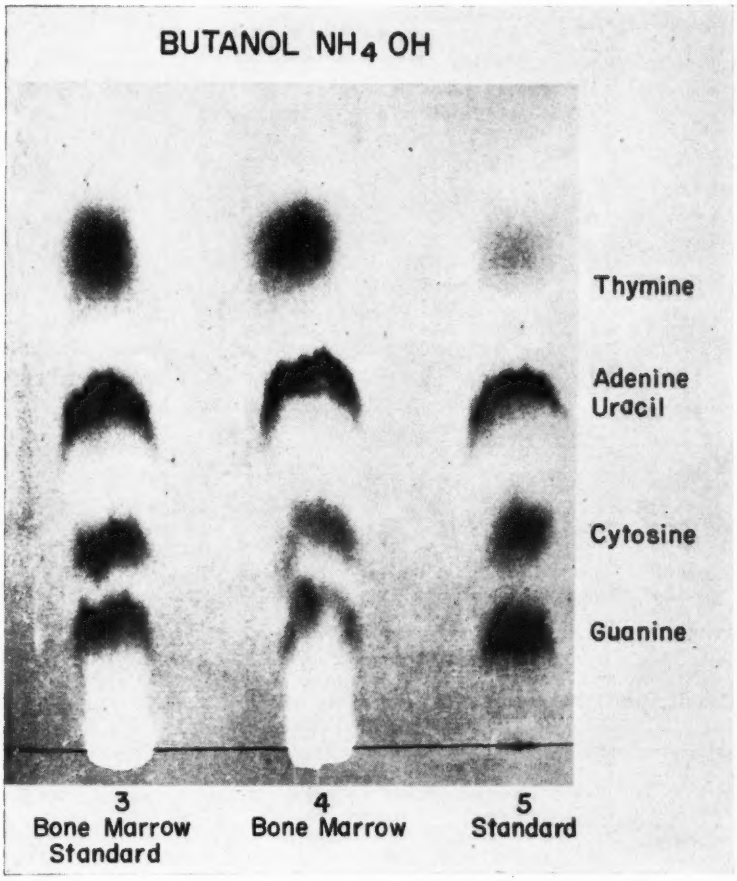


Fig. 2.—A paper chromatogram, photographed in ultraviolet light, demonstrating the separation of thymine, cytosine, guanine, and a mixture of adenine and uracil by the alkaline-butanol system.

TABLE I. RECOVERIES OF PURE STANDARDS OF PURINES AND PYRIMIDINES BY BUTANOL-AMMONIA AND BUTANOL-HYDROCHLORIC ACID SYSTEMS

| STANDARD | SYSTEM | NO. OF DETERMINATIONS | PER CENT RECOVERED | STANDARD DEVIATION |
|---|---|---|---|---|
| Thymine | Butanol-HCl | 44 | 93.6 | 8.0 |
| Uracil | Butanol-Hcl | 43 | 90.7 | 7.4 |
| Thymine | Butanol-$NH_4OH$ | 6 | 102.0 | 5.0 |
| Adenine | Butanol-$NH_4OH$ | 4 | 90.5 | 4.0 |
| Cytosine | Butanol-$NH_4OH$ | 6 | 89.0 | 9.7 |
| Guanine | Butanol-$NH_4OH$ | 6 | 83.2 | 15.0 |

The results are expressed as molar ratios: RNA to DNA, U to T, T to total bases, as we cannot as yet determine the absolute quantities per bone marrow cell.

The chromatographic method is reasonably accurate. Recoveries of pure standards in both systems are shown in Table I and duplicate determinations on bone marrow specimens are given in Table II.

TABLE II. RESULTS OF DUPLICATE DETERMINATIONS OF BONE MARROW ANALYSES FOR PURINES AND PYRIMIDINES

| ACID-BUTANOL SYSTEM | | | | | |
|---|---|---|---|---|---|
| T (α) | | U (α) | | U/T (MOLAR RATIO) | |
| 1.02 | 1.16 | 0 | 0.144 | 0.0 | 0.14 |
| 2.04 | 2.01 | 0.68 | 0.63 | 0.37 | 0.35 |
| 8.25 | 8.73 | 0.97 | 1.36 | 0.13 | 0.176 |
| 3.9 | 4.1 | 0.54 | 0.36 | 0.155 | 0.10 |
| 0.36 | 0.33 | 0.13 | 0.20 | 0.42 | 0.70 |
| 2.58 | 2.7 | 0.35 | 0.35 | 0.15 | 0.13 |
| 5.2 | 6.0 | 0.68 | 1.03 | 0.15 | 0.19 |
| 4.2 | 4.3 | 0.76 | 0.81 | 0.20 | 0.21 |
| 5.85 | 5.87 | 0.52 | 0.44 | 0.10 | 0.09 |
| 3.2 | 4.0 | – | – | – | – |
| 1.75 | 2.11 | 0.22 | 0.35 | 0.14 | 0.19 |
| 2.6 | 2.8 | 0.30 | 0.22 | 0.09 | 0.10 |
| 3.2 | 2.4 | 0.07 | 0.12 | 0.34 | 0.30 |
| 3.3 | 3.1 | 0.51 | 0.51 | 0.18 | 0.19 |
| 1.6 | 1.7 | 0.54 | 0.43 | 0.31 | 0.41 |
| 3.65 | 3.63 | 0.90 | 1.05 | 0.28 | 0.33 |
| 1.31 | 1.4 | 0.22 | 0.15 | 0.51 | 0.32 |
| – | – | – | – | 0.07 | 0.15 |

| ALKALINE-BUTANOL SYSTEM | | | | | | | |
|---|---|---|---|---|---|---|---|
| T | | A AND U | | C | | G | |
| 1.45 | 1.15 | 2.85 | 2.70 | 2.25 | 2.05 | 3.05 | 2.55 |
| 0.75 | 0.80 | 0.46 | 0.52 | 1.00 | 0.90 | 0.60 | 0.40 |
| 0.95 | 1.02 | 1.27 | 1.25 | 0.95 | 0.86 | – | 0.98 |
| – | 0.89 | 1.23 | 1.28 | 1.02 | 0.93 | 1.26 | 1.00 |
| 1.75 | 1.39 | 2.55 | 2.35 | 1.35 | 1.43 | 1.90 | 2.03 |
| 2.4 | 2.25 | 3.94 | 3.99 | 2.89 | 2.83 | 1.93 | 2.01 |
| 3.79 | 3.36 | 4.98 | 4.75 | 2.15 | 2.05 | 2.92 | 2.86 |

TABLE III. STUDIES OF NUCLEIC ACIDS IN PERNICIOUS ANEMIA. CHANGES IN THE NUCLEIC ACIDS, URACIL, AND THYMINE OF THE BONE MARROW AND IN THE CELLULAR MORPHOLOGY IN RESPONSE TO TREATMENT

| NO. | DIAGNOSIS | THERAPY | % MEGALOBLASTS | RNA/DNA | U/T | %T |
|---|---|---|---|---|---|---|
| 1 | Sprue | none | 75 | 1.0 | 0.27 | – |
| | | A.A. 1000 mg. × 10 | 49 | 0.7 | 0.16 | – |
| | | $B_{12}$ 0.25γ × 10 | 23 | – | 0.16 | – |
| | | $B_{12}$ 1γ × 10 | 3 | 0.33 | – | – |
| 2 | P.A. | none | 74 | 1.0 | 0.41 | 14 |
| | | $B_{12}$ 5γ × 3 | 32 | 0.65 | 0.29 | 20 |
| | | $B_{12}$ 5γ × 25 | 0 | – | 0.07 | 26 |
| 3 | P.A. | none | 66 | 0.76 | 0.41 | – |
| | | $B_{12}$ 15γ × 10 | 0 | 0.48 | 0.16 | – |
| 4 | P.A. | none | 92 | 0.89 | 0.37 | – |
| | | $B_{12}$ 15γ × 3 | 54 | 0.42 | 0.21 | – |
| | | $B_{12}$ 15γ × 10 | 0 | 0.43 | – | – |
| 5 | P.A. | none | 80 | 0.61 | 0.33 | – |
| | | none | 88 | 0.66 | 0.33 | – |
| | | F.A. ½ mg. × 10<br>F.N.A. 1 mg. × 10 | 24 | 0.48 | 0.29 | – |

A.A. = ascorbic acid; $B_{12}$ = vitamin $B_{12}$; F.A. = folic acid; F.N.A. = folinic acid.

RESULTS

In the control group the RNA/DNA ratios were 0.316 ± 0.073,* the U/T ratios 0.133 ± 0.027. In the patients with megaloblastic anemia before treatment, the RNA/DNA ratios were 0.835 ± 0.141, the U/T ratios 0.316 ± 0.078 or about two and one-half times higher than the controls (Fig. 3). After treatment these ratios fell to normal. When all of the bases were determined, the percentage of thymine was low in the megaloblastic marrows and rose after treatment, while uracil was high and fell.

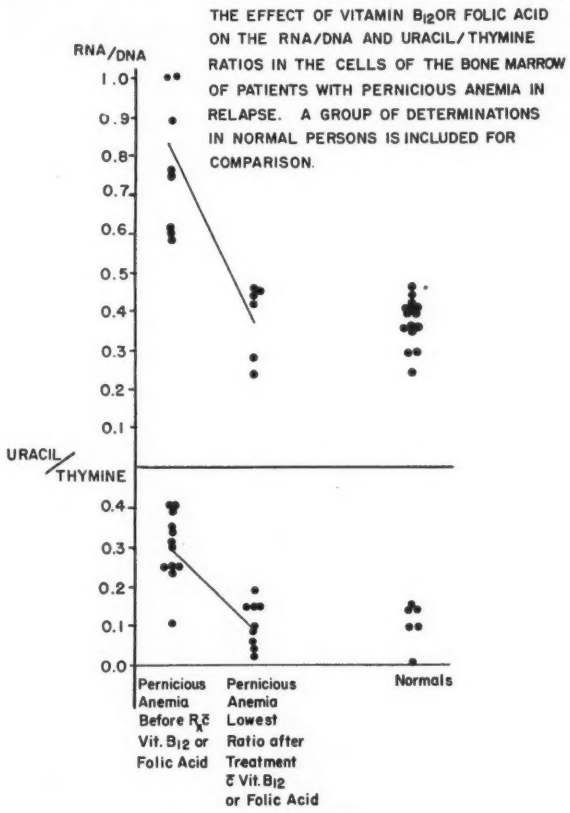


Fig. 3.

Concurrent with the fall in the RNA/DNA and U/T ratios and the changes in T and U percentages the expected shift from megaloblast to normoblast occurred in the marrow as illustrated in Tables III and IV. Before therapy the percentage of the pernicious anemia type of cell was usually above 70 per cent. As treatment progressed the percentage of this type of cell fell until the marrow was completely normal with no megaloblasts present.

In many cases the change in ratios preceded the rise in reticulocytes as illustrated in Fig. 4. Marrow specimens in which the major maturation defect was found in the myeloid series had comparable though less strikingly elevated ratios.

*Standard deviation.

TABLE IV. STUDIES OF NUCLEIC ACIDS IN PERNICIOUS ANEMIA. CHANGES IN THE NUCLEIC ACIDS, URACIL, AND THYMINE OF THE BONE MARROW AND IN THE CELLULAR MORPHOLOGY IN RESPONSE TO TREATMENT (CONT'D)

| NO. | DIAGNOSIS | THERAPY | % MEGALO-BLASTS | RNA/DNA | U/T | %T |
|---|---|---|---|---|---|---|
| 6 | P.A. | none | 80 | – | 0.3 | 15 |
| | | F.A. 10 mg. × 10 | 0 | – | 0.05 | 29 |
| 7 | P.A. | none | 76 | – | 0.31 | 22 |
| | | $B_{12}$ 5γ × 4 | 0 | – | 0.03 | 28 |
| 8 | P.A. | none | 48 | – | 0.12 | – |
| | | $B_{12}$ 5γ × 6 | 0 | – | 0.08 | – |
| 9 | P.A. | none | 85 | – | 0.26 | 24 |
| | | $B_{12}$ 10γ × 10 | 0 | – | 0.19 | 25 |
| 10 | P.A. | none | 66 | – | 0.26 | 21 |
| | | A.A. 1000 mg. × 10 | 76 | – | 0.29 | – |
| | | none | 71 | – | 0.22 | 22 |
| | | $B_{12}$ 0.25γ × 10 | 0 | – | 0.10 | – |
| 11 | P.A. | none | 71 | 0.75 | 0.39 | – |
| | | $B_{12}$ 30γ × 4 | 2 | 0.34 | – | – |
| | | $B_{12}$ 30γ × 10 | 0 | 0.27 | – | – |

A.A. = ascorbic acid; $B_{12}$ = vitamin $B_{12}$; F.A. = folic acid; F.N.A. = folinic acid.

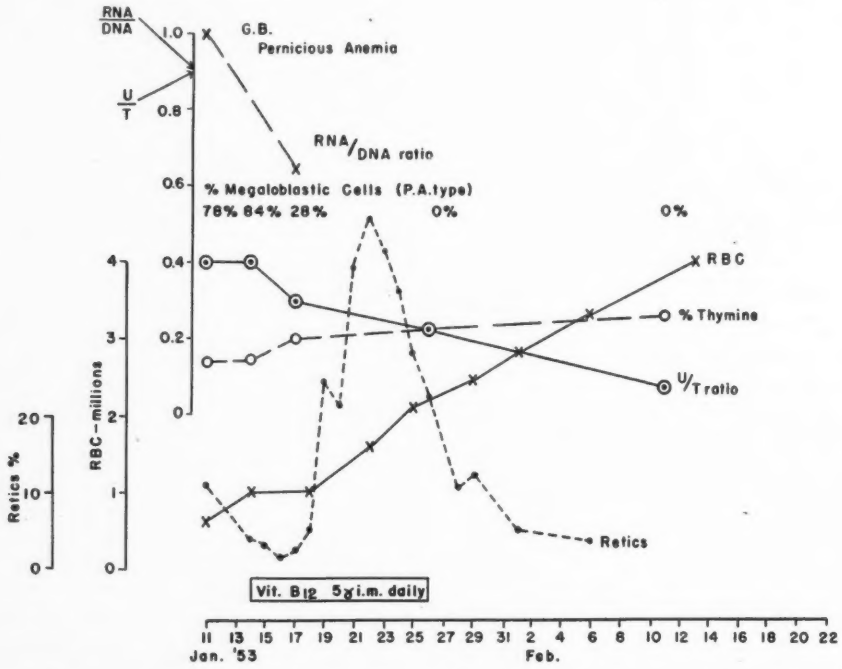


Fig. 4.—Graph illustrating the relationship of the hematologic response of a patient with pernicious anemia to the changing RNA/DNA ratio and thymine concentration in the bone marrow cells. The fall in these ratios and the rise in thymine concentration paralleled the change in the marrow from megaloblastic to normoblastic type and preceded the reticulocyte response to vitamin $B_{12}$.

## DISCUSSION

RNA occurs primarily in the cytoplasm and the nucleolus of the cell. It is involved in protein synthesis[5]; as long as active protein formation takes place, RNA metabolism is active. DNA, which is contained in the cell nucleus, is more

stable with a slower turnover rate.[6] It is necessary for cell division. When mitosis is active, DNA is high, especially during the prophase, while RNA is increased when there is cell growth with protein formation.

The effect of the hematopoetic vitamins on the formation of nucleic acids and their purine and pyrimidine base constituents has been studied by various means. Biochemical work recently reviewed by Welch and Nichol[7] indicates that vitamin $B_{12}$ and the folic acid-ascorbic acid system act as transfer agents for single C units. It had been shown that folic acid is involved in the introduction of the 2 and 8 C atoms into the purine ring and that it controls the addition of a formate (or methyl) group to uracil riboside to form thymine desoxyriboside. Vitamin $B_{12}$ is also an essential factor involved in the biosynthesis of thymine and thymidine[8] and may be concerned with the formation of desoxyribose phosphate esters. Microbiologic evidence also indicates a close relationship between the hematopoetic vitamins and the purine and pyrimidine bases. In the presence of purines, thymine or thymidine will replace folic acid as growth factors for *Streptococcus faecalis* R and *Lactobacillus casei*[9, 10] and these pyrimidines will also overcome the inhibitory effect of folic acid antagonists.[11] If media used in growing the organism is deficient in folic acid, DNA is formed slowly. This defect is overcome by the addition of large amounts of thymine; formation of RNA is increased also by this addition.[12]

Various desoxyribosides will replace vitamin $B_{12}$ in promoting growth of certain organisms, and conversely $B_{12}$ will stimulate most organisms which respond to thymidine. Thus there seems to be an interchangeability of vitamin $B_{12}$ and thymidine as growth factors for these organisms which are chiefly of the lactic acid group.[13]

Cytochemical studies on bone marrow from pernicious anemia patients have shown that the granular state of the RNA of the erythroblast becomes homogeneous after injection of vitamin $B_{12}$ or folic acid.[14] When the liver cells of rats are damaged by $CCl_4$, RNA is depleted; vitamin $B_{12}$ in massive doses protects this nucleic acid.[15] In vitamin $B_{12}$-deficient rats there is less RNA and DNA in the liver,[16] and less RNA in anterior horn and autonomic ganglion cells[17] than in rats given vitamin $B_{12}$. After vitamin $B_{12}$ administration, the concentration of RNA rose and, in some instances, the concentration of DNA also rose slightly.

While work in our laboratory was in progress, reports on the content of nucleic acid phosphorus in bone marrow cells were published.[18, 19] These show that the amounts of both RNA and DNA phosphorus in the cells are high during relapse of pernicious anemia and decrease after the administration of folic acid or vitamin $B_{12}$. RNA decreases to a much greater extent than DNA, resulting in changes in the RNA/DNA ratios similar to those reported in this paper. Spectrophotofluorometric determinations of cellular DNA under these conditions have not demonstrated any change in concentration.[20]

All of these studies indicate that the hematopoietic vitamins affect nucleic acid metabolism. The effects observed are more striking in regard to RNA than DNA, but are not consistently in the same direction. Only slight changes have been observed in DNA concentration, yet the microbiologic evidence indicates

a much more fundamental relationship to thymine and its relatives, components of DNA, than to the other purine and pyrimidine bases. Since the DNA content of cells of any species seems to be a very constant value, except for the increase that occurs during mitotic prophase, a change in DNA concentration in response to a deficiency of folic acid or vitamin $B_{12}$ might not occur. It is more likely that the net result of such a deficiency would be deceleration of mitosis and ultimate cell death rather than decrease in cellular DNA content. Cells in such a resting stage could not mature normally and the specific cellular characteristics of RNA under these conditions would become apparent. With replacement of the deficient vitamin and re-establishment of mitotic activity and maturation, the normal changes in RNA for the particular cell type would be observed. In one case this might be an increase in RNA content; in another, it might be a considerable decrease.

Our studies indicate that in the mixed nucleic acids of bone marrow from patients with pernicious anemia in relapse, there is more RNA compared to DNA and more uracil compared to thymine or total bases than in normal marrow. For this reason the RNA/DNA and U/T ratios are high. These abnormal relationships are corrected by treatment with the hematopoietic vitamins. The concentrations of RNA and uracil fall, while concentrations of DNA and thymine increase. These changes are consistent with the alterations that occur in the morphology of the cells, namely, a decrease in cytoplasmic basophilia and an increase in mitotic activity and in the maturity of the nuclear chromatin. Although the absolute amounts of the nucleic acids or their bases have not as yet been determined per cell, our results are consistent with the hypothesis that, in hematopoetic tissues, vitamin $B_{12}$ and the folic acid-ascorbic acid systems initiate chemical reactions that favor the formation of thymine and its desoxyribotides and the depletion of uracil and its nucleotides.

There is suggestive evidence, also, that a greater percentage change takes place in the pyrimidine concentrations than in the nucleic acid concentrations. This may indicate that a qualitative effect on the composition of the nucleic acids is initiated by these vitamins.

There has been a great deal of evidence already published indicating that the tetranucleotide theory of nucleic acid structure is invalid. Our data offer additional proof. If it were correct there would be one molecule of uracil per molecule of RNA, and one of thymine per molecule of DNA. The RNA/DNA and U/T ratios would be identical. This is obviously not the case, either in normal or megaloblastic marrows.

## SUMMARY AND CONCLUSIONS

A method for the determination of purine and pyrimidine bases by paper chromatography has been modified and adapted to the study of human bone marrow.

The ratios of RNA/DNA, U/T, and T/total purine and pyrimidine bases have been determined in a control group and a group of patients with megaloblastic anemia before, during, and after therapy with folic acid, vitamin $B_{12}$, and ascorbic acid.

The RNA/DNA and U/T ratios are higher than normal in patients with megaloblastic anemia; the relative amount of thymine is lower than normal.

After adequate treatment with the hematopoietic vitamins the RNA/DNA and U/T ratios fall and the relative amount of thymine rises to normal as the bone marrow becomes normal cytologically.

Further evidence has been given that the tetranucleotide theory of nucleic acid composition is incorrect.

We wish to thank Dr. Milan A. Logan, Department of Biochemistry, and Dr. Victor P. Whittaker, Department of Physiology, College of Medicine, University of Cincinnati for many helpful suggestions. The vitamins used in these experiments were generously supplied by Dr. Elmer Alpert, Merck and Co., Inc., and by Dr. James Ruegsegger of Lederle Laboratories.

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# THE TIME FACTOR IN ATHEROSCLEROSIS

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## INTRODUCTION

CLINICALLY, atherosclerosis is a chronic disease characterized by long-term symptomatology and morbidity. If, however, it were shown that the intimate pathogenesis of the disease is a short-term process measured in weeks or days rather than months and years, important implications would arise, especially in so far as prevention of the disease is concerned.

There is considerable evidence that this is the case. Holman,[1, 2] from human autopsy material, concluded that the pathologic deposition of lipid material in blood vessel walls occurred in "crops" and that the long-term clinical effects were a function of the amount deposited in these short-term episodes. Bevans[3] and Pollak[4] were able, by the parenteral administration of artificial lipid emulsions into experimental animals, to produce in a matter of days vessel wall atheromata that were indistinguishable from early human lesions. Bragdon[5] extended these observations by finding that the intravenous injection into normal rabbits of hyperlipemic serum taken from animals previously fed cholesterol resulted in typical lesions as early as twenty-four hours later.

It is the purpose of this paper to report evidence supporting and extending some of these findings.

## MATERIALS AND METHODS

A group of thirty rabbits, averaging just over 2 kilograms in weight, was used in the first experiment. Six were autopsied as controls; the remaining animals were placed on a high-cholesterol diet containing a daily portion of 6 Gm. of cholesterol and 6 Gm. of salad oil mixed with Purina rabbit chow. The diet was well tolerated by the animals and a steady weight gain was obtained.

Six animals were autopsied at intervals of five, eleven, eighteen, and twenty-five days after beginning the diet. Blood specimens were taken for determination of total cholesterol, cholesterol esters,[6] and lipid phosphorus[7] just prior to autopsy. Because each group of six animals was selected for autopsy from animals with highest prevailing serum lipid levels, there was a marked tendency to relatively low serum lipid levels in those remaining groups on the diet for the eighteen- and twenty-five-day periods.

At autopsy, the presence or absence of gross lesions of the aorta was noted. In their absence, random microscopic sections were taken from two areas of the aorta, one close to the aortic valves, and the other in the descending arch. Frozen sections were stained with oil-red-O and the degree of microscopic atherosclerosis estimated on a 0 to 4+ scale (1+ being the earliest recognizable lesion involving the endothelium, 4+ being the presence of sudanophilic material throughout the intima and penetrating down into the media).

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This investigation was supported by a grant from the National Heart Institute, United States Public Health Service.

Received for publication, Feb. 1, 1954.

It should be noted that the limitations of the staining technique imposed a need for disciplined interpretation of material. The distribution and histologic appearance of the dye in some sections, especially where adjacent to traumatized tissue, suggested that this material did not represent atheromatous lesions. These deposits were felt to consist of either precipitates of dye in areas not containing lipid or the spread of a nonatheromatous lipid over the surface of the section, for example, from the adventitia. Accordingly, all deposits which did not show a characteristic fine homogeneous distribution were excluded as artefacts.

In the second experiment, a group of rabbits fed a high-cholesterol diet, as described previously, was kept as a source of hyperlipemic serum. At intervals, using sterile technique, these animals were bled by heart puncture. Their sera were separated, pooled, crystalline penicillin 200 units per milliliter added, and stored in a refrigerator. Chemical determinations showed no appreciable change in the levels of cholesterol or lipid phosphorus in this pooled serum when stored as long as one week.

TABLE I. DIET SCHEDULE, SERUM LIPID LEVELS, AND DEGREE OF ATHEROSCLEROSIS IN TWENTY-FOUR RABBITS FED HIGH-CHOLESTEROL (6 GM. DAILY) DIET AND SIX RABBITS FED CONTROL DIET

| NO. DAYS ON DIET | PREAUTOPSY SERUM LEVELS | | DEGREE OF MICROSCOPIC ATHEROSCLEROSIS |
|---|---|---|---|
| | TOTAL CHOLESTEROL (MG. PER 100 ML.) | LIPID PHOSPHORUS (MG. PER 100 ML.) | |
| 0 | 62 | 3.3 | 0 |
| 0 | 58 | 3.4 | 0 |
| 0 | 61 | 3.7 | 0 |
| 0 | 66 | 3.6 | 0 |
| 0 | 70 | 3.8 | 0 |
| 0 | 53 | 3.1 | 0 |
| 5 | 312 | 14.0 | 0* |
| 5 | 77 | 3.7 | 0* |
| 5 | 183 | 5.8 | 0 |
| (died) | — | — | — |
| 5 | 94 | 14.0 | 0 |
| 5 | 462 | 12.0 | 0* |
| 11 | 1,103 | 16.0 | 2+ |
| 11 | 1,270 | 16.6 | 2+ |
| 11 | 2,847 | 27.4 | 2+ |
| 11 | 1,334 | 17.4 | 0* |
| 11 | 769 | 13.9 | 1+ |
| 11 | 1,924 | 21.2 | 2+ |
| 18 | 538 | 10.0 | 0 |
| 18 | 434 | 12.0 | 0 |
| 18 | 1,680 | 21.4 | 2+ |
| 18 | 564 | 11.4 | 2+ |
| 18 | 1,462 | 18.9 | 2+ |
| 18 | 1,231 | 17.7 | 1+ |
| 25 | 500 | 10.0 | 1+ |
| 25 | 600 | 12.4 | 3+ |
| 25 | 1,010 | 18.0 | 3+ |
| 25 | 627 | 11.3 | 3+ |
| 25 | 241 | 6.4 | 2+ |
| 25 | 465 | 11.5 | 1+ |

*Possible but not definite lesions present.

In the first part of this experiment, normal rabbits were given single injections into an ear vein of approximately 50 ml. of this hyperlipemic serum (containing more than 2,000 mg. total cholesterol per 100 ml.). Two animals were sacrificed at intervals of 1, 6, and 24, and 48 hours after the injection. Bloods were drawn at intervals prior to sacrifice to determine levels of serum cholesterol and lipid phosphorus. The degree of microscopic atherosclerosis was determined as described previously.

In the second experiment, a group of rabbits fed a high-cholesterol diet, as described serum were given to normal animals. One animal was autopsied after 4, 8, 11, 12, 16, 20, 24, and 30 days of such daily injections. Again bloods were drawn before sacrifice. The degree of atherosclerosis was determined at autopsy.

## RESULTS

1. The oral administration of the high-cholesterol diet resulted in an early rise in the level of the measured serum lipids, and in the early appearance of definite microscopic lesions at eleven days. The level of measured serum lipids and the degree of atherosclerosis found are shown in Table I.

2. *A.* Single intravenous injection of pooled hyperlipemic serum from donor rabbits resulted in possible minimal microscopic lesions in twenty-four hours and forty-eight hours. These possible atheromata, failing to meet the criteria for definite lesions, are reported negative in Table II.

*B.* Repeated daily injections of hyperlipemic serum produced definite microscopic lesions at 4, 11, 12, 16, 24, and 30 days; possible lesions were noted at eight days. Gross atheromata were apparent in the thirty-day aorta. Blood lipid levels before sacrifice and pathologic findings for Parts 2 *A* and *B* are summarized in Table II.

TABLE II. INJECTION SCHEDULE, SERUM LIPID LEVELS AND DEGREE OF ATHEROSCLEROSIS IN SIXTEEN RABBITS GIVEN HYPERLIPEMIC SERUM INTRAVENOUSLY

| NO. OF INJECTIONS | INTERVAL UNTIL AUTOPSY | PREAUTOPSY SERUM LEVELS | | DEGREE OF MICROSCOPIC ATHEROSCLEROSIS |
|---|---|---|---|---|
| | | TOTAL CHOLESTEROL (MG. PER 100 ML.) | LIPID PHOSPHORUS (MG. PER 100 ML.) | |
| 1 | One hour | 211 | 5.3 | 0 |
| 1 | One hour | 220 | 5.2 | 0 |
| 1 | Six hours | 159 | 5.0 | 0 |
| 1 | Six hours | 222 | 5.0 | 0 |
| 1 | Twenty-four hours | 190 | 4.8 | 0 |
| 1 | Twenty-four hours | 159 | 4.6 | 0* |
| 1 | Forty-eight hours | 160 | 4.3 | 0* |
| 1 | Forty-eight hours | 188 | 4.0 | 0* |
| 4 | Four days | 710 | 15.2 | 2+ |
| 8 | Eight days | 1,216 | 16.1 | 0* |
| 11 | Eleven days | — | — | 3+ |
| 12 | Twelve days | 2,418 | 19.0 | 4+ |
| 16 | Sixteen days | 1,721 | 17.8 | 3+ |
| 20 | Twenty days | 2,665 | 18.6 | 3+ |
| 24 | Twenty-four days | 2,010 | 17.5 | 4+ |
| 30 | Thirty days | 1,630 | 18.6 | 4+ |

*Possible but not definite lesions present.

It should be noted that in both experiments serial sections through a larger area of the aortas possibly would have revealed earlier occurring lesions.

## DISCUSSION

According to currently held concepts, there are two factors in the pathogenesis of atherosclerosis. One is qualitative or quantitative abnormalities in the circulating lipids, and the others, local conditions (increased luminal pressure, disturbed integrity of vessel wall, and so forth) determining localization, distri-

bution, and extent of lesions. Since in the present state of our knowledge little can be done about these local factors, therapeutic efforts have been directed toward adjustment of circulating lipids.

From the data reported in this paper, it appears that the necessary lipid abnormalities need be present only a short time for atheromatous lesions to develop. It follows that any transient abnormality in serum lipids during the lifetime of an individual might result in a "crop" of atheromatous lesions, some of which may lead to long-term clinical atherosclerosis.

## SUMMARY

Rabbits fed a daily diet containing 6 Gm. of cholesterol and 6 Gm. of salad oil will develop definite minimal microscopic atherosclerotic lesions in as early as eleven days.

Normal rabbits given a single intravenous injection of hyperlipemic serum taken from animals previously fed cholesterol-salad oil develop possible minimal atherosclerotic lesions in twenty-four to forty-eight hours.

Normal rabbits given daily intravenous injections of hyperlipemic serum taken from donor animals develop definite minimal lesions as early as after four injections and gross lesions after thirty injections.

Evidence is presented that the pathogenesis of atherosclerosis may be a short-term process measured in hours and days once the necessary condition of serum lipids obtains.

Acknowledgment is made of the valued assistance of Miss Stephanie J. Ilka for Laboratory chemistry determinations, and Mrs. Elizabeth S. Hausner for preparation of microscopic sections.

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# THE PERSISTENCE IN THE BLOOD OF THE RADIOACTIVE LABEL OF ALBUMIN, GAMMA GLOBULINS, GLOBULINS OF INTERMEDIATE MOBILITY STUDIED WITH $S_{35}$ AND PAPER ELECTROPHORESIS: METHODS AND PRELIMINARY RESULTS

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## INTRODUCTION

IN THE shift in emphasis from levels (concentrations) of biologically important substances in blood to the processes responsible for maintenance or change in these levels (rate of synthesis, rates of degradation or removal), the study of proteins has lagged behind the study of fats, steroids, carbohydrates, and simpler molecular species.

This is not ascribable to the size or the submicroscopic complexity of protein molecular fine structure. In part it may be due to tediousness of measuring isotopes hitherto used in protein tagging; in part due to difficulty in separating complex mixtures of proteins into reproducible fractions. For reproducibility is a minimum requirement for such work when purity of molecular species in the strict thermodynamic sense is rarely attainable.

London's studies[1] (the only published measurements on human plasma proteins until the last two years) fractionated by free electrophoresis in the Tiselius type cell. This yields the fastest, albumin, and slowest, gamma globulin, in only fair purity by electrophoretic criteria and, indeed, in only fair reproducibility.[2] It does not readily yield specimens of the globulin components intermediate in mobility.

London used heavy nitrogen, as did Schoenheimer and associates[3] in their classic studies on animal proteins. Measurement with a mass spectrometer is expensive and time-consuming, demands a high degree of technical skill, and is as little suited as is fractionation by free electrophoresis to studies in which multiple determinations throughout a disease process are wanted.

Since then, several methods of protein fractionation which lend themselves to multiple serial determinations with reasonable speed and reproducibility have appeared. Notable is the small scale ethanol-water method (introduced by Cohn and associates,[5] Lever and co-workers,[6] and explored clinically by Eder,[7] Barr,[8] and others), and the method of paper electrophoresis as worked out by Kunkel and Tiselius.[9]

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Received for publication, Feb. 6, 1954.
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The labeling of proteins with radioactive isotopes has also greatly facilitated counting. If the protein fraction to be studied is labeled outside the body as in the instance of $I_{131}$ labeled albumin, the question of whether any stage in the in vitro treatment changes the manner (and thus perhaps the rate) in which the fraction is broken down within the body. Sterling,[10, 11] in his studies on normals and cirrhotics, made a serious attempt to rule this possibility out. Such an objection is avoided when a patient synthesizes his own proteins from orally administered, labeled amino acids.

Table I gives a representative group of values from the current literature for human plasma protein fractions. The table indicates methods of fractionation, isotopic labeling, and clinical conditions.

The fact that the apparent turnover times for $I_{131}$ labeled albumin free of "rapidly degraded components," as measured by Berson and co-workers,[12] are close to the literature values for albumin synthesized by the patient, whereas values for $I_{131}$ labeled albumin containing "rapidly degraded components" are close to literature values of previous workers with $I_{131}$, emphasizes the possibility that changes to the protein by in vitro labeling can be as important a factor in apparent turnover time as is metabolic status of the patient.

Because $S_{35}$ in dosage currently allowed, by reason of low counts, has presented difficult methodological problems, and because in the process of solving these problems we have come upon some apparent turnover times far outside the previously reported range, we present here the essentials of the combined methods and these preliminary results.

## METHODS

*Free Electrophoresis.*—This has been carried out and diagrams resolved as previously published.[2]

*Paper Electrophoresis.*—The methods described by Kunkel and Tiselius[9] have been adapted to needs of quantitative extraction and radioactivity determinations thus: Analyses are carried out in sodium diethylbarbiturate buffer, pH 8.8, ionic strength 0.1. Duration of runs varies from twenty to twenty-four hours at currents between 18 and 20 Ma. and starting voltages between 140 and 150 in a cold room at approximately 5° C. The pH of 8.8 has been selected because in our hands it gives clearer separations than achieved at either higher or lower hydrogen-ion concentrations.

Four rectangles of Munktell No. 20 filter paper 30 by 12.5 cm. are superimposed in a pack to form the supporting medium. Undialyzed, undiluted serum is applied in a row of three 0.33 ml. spots approximately 10 cm. from the cathode end of the paper pack. This comparatively large amount of serum is used in order to obtain fractions with sufficient radioactivity over background to permit quantitative interpretation of biologic decay curves. Larger amounts of serum (up to 1.5 ml. per spot) have given good separations when the distribution of electrophoretic components by free electrophoresis has approximated the normal. In pathologic sera with high globulin levels, however, or in sera where the albumin component is artificially elevated by the addition of purified albumin, spots made with greater than 0.33 ml. have shown progressively poorer separations.

In Fig. 1, *A* is illustrated a paper electrophoresis with adequate separation; in Fig. 1, *B*, one with inadequate separation for our purposes. Runs similar to Fig. 1, *B* have been discarded and analyses repeated.

TABLE I. REPRESENTATIVE ISOTOPICALLY MEASURED "TURNOVER TIMES" OF PLASMA PROTEIN FRACTIONS IN MAN*

| INVESTIGATOR | ADMINISTERED LABEL AND ROUTE | FRACTIONATION PROCEDURE | CLINICAL CONDITION | APPARENT TURNOVER TIMES (AVERAGES) ($T_{1/2}/LOG_e 2$) | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | ALB | MIXTURE ALB + $\alpha$ | MIXTURE $\alpha$ + $\beta$ | MIXTURE $\beta$ + $\gamma$ | $\gamma$ | FIB |
| London[1] | $N_{15}$ glycine Oral | Free electrophoresis | Normal | 29 | 17 | | 15 | 27 | |
| Volwiler and associates[13] | $S_{35}$ dl-cystine Oral | Chemical | Normal | 25 | | 6 | | 39 | |
| | | | Advanced cirrhosis | 30 | | 20 | | 36 | |
| Berson and associates[12] | $I_{131}$ albumin Intravenously | None | | | | | | | |
| | a. Free of rapidly degraded components | | Normal | 26 | | | | | |
| | | | Laennec's cirrhosis | >31 | | | | | |
| | b. With rapidly degraded components | | Misc. diseases | 19 | | | | | |
| | | | Laennec's cirrhosis | 21 | | | | | |
| Sterling[10, 11] | $I_{131}$ human albumin Intravenously | None | Normal | 15 | | | | | |
| | | | Laennec's cirrhosis | 19 | | | | | |
| Freinkel and associates[15] | $I_{131}$ human albumin Intravenously | None | Normal | 13 | | | | | |
| Schoenberger and associates[17] | $I_{131}$ albumin Intravenously | None | Laennec's cirrhosis | 14 | | | | | |
| Vaughan and associates[14] | $I_{131}$ $\gamma$-globulin Intravenously | Chemical | Normal | | | | | 10 | |
| | | | Active rheumatoid arthritis | | | | | 9 | |
| Madden and associates[16] | $S_{35}$ di-methionine Oral | Washed, dilute clots | Normal | | | | | | 8 |

*Since the preparation of this table, Eisenmenger and Slater (J. Clin. Invest. 32: 564, 1953) have published the following apparent turnover times for $I_{131}$ labeled albumins and gamma globulins in normals and in patients with cirrhosis:

Albumin for normals and cirrhotics—13½ through 16½ days

Gamma Globulins

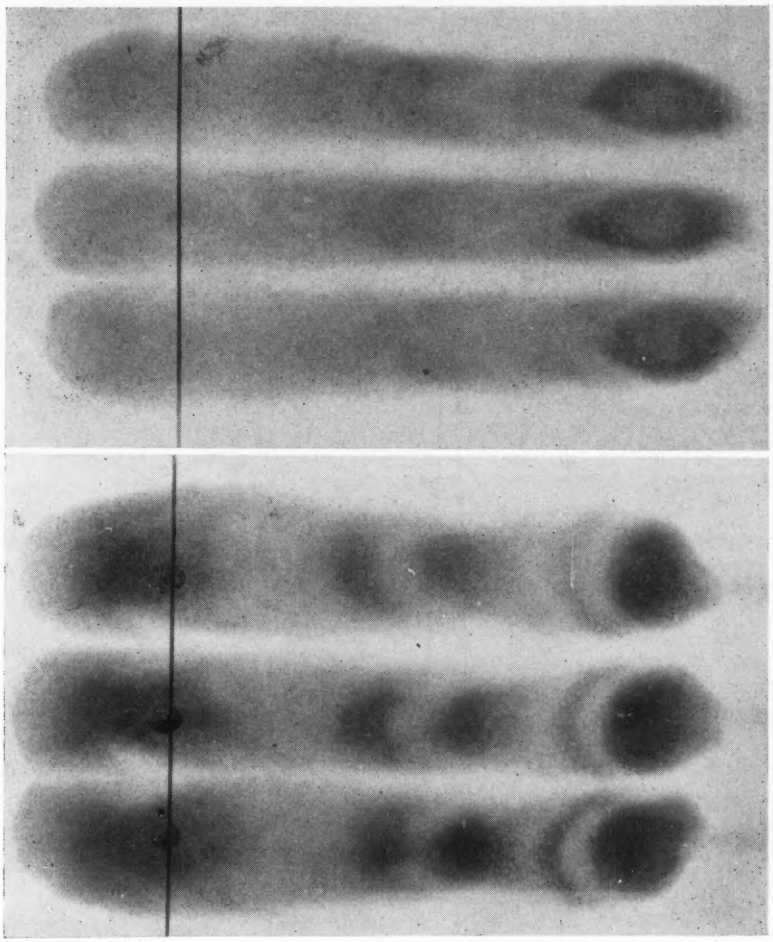
Normals—16½ through 27 days

Cirrhotics—8½ through 18½ days

(These figures are obtained by dividing the half-lives cited by $\log_e 2$ to obtain the apparent turnover time.)

Extreme care of handling the paper from start to finish is a prime requisite to good results. Any mechanical damage to the integrity of the paper structure results in streaky, uneven flow. Moreover, in setting up or in removing the paper at the end of the run, any slipping of the glass plates can result in disturbance of the boundaries and consequent streaking.

*A.*



*B.*

Fig. 1.—*A* and *B*, Photographs of paper electrophoretic runs, dyed as described in text, illustrating (*A*) adequate (*B*) inadequate separation of components. Vertical line indicates starting position; component furthest to right is albumin, to left is γ-globulins.

*Identification of Components.*—At the end of the run, the top rectangle of filter paper is carefully lifted off and dyed in a solution of bromphenol blue dissolved in 95 per cent ethanol saturated with $HgCl_2$ for seven minutes, followed by four washings with 0.5 per cent acetic acid. This strip is then blotted and air-dried. A carbon tracing is made by superimposing the dyed strip on the remaining three strips, and the spots containing the separated electrophoresis components are excised using this tracing as a guide. It is essential for quantitative elution of the protein in these components that excision be carried out with dispatch. If delayed so that paper approaches dryness, subsequent elution of the alpha and beta globulins is rarely quantitative.

*Quantitative Elution of Protein in Components and Formation of Film for Weighing and Measurement of Radioactivity.*—The excised portions of paper corresponding to the separated components are dropped in about 40 ml. of 0.1 N $NH_4OH$, fragmented thoroughly with a small glass stirring rod, and allowed to stand for twenty-four hours at room temperature.

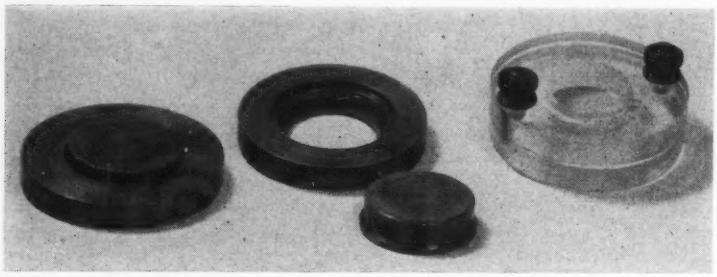
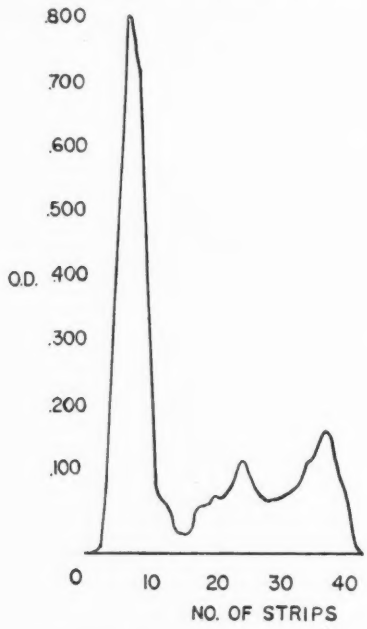


Fig. 2.—Photographs of brass planchette and ring and plexiglass rings (used to keep films flat during drying for reproducibility in radioactivity measurements.)




DISTRIBUTION OF COMPONENTS (%)

| | ALB. | $\alpha_1$ | $\alpha_2$ | $\beta$ | $\gamma$ |
|---|---|---|---|---|---|
| PAPER | 53 | 4 | 7 | 11 | 25 |
| FREE | 60 | 3 | 5 | 12 | 20 |

Fig. 3.—Representative comparison of distribution of electrophoretic components obtained from optical density—distance plot of extracted dyed paper electrophoresis (illustrated) and from schlieren diagram of free electrophoresis (not illustrated).

The eluate is removed from filter paper by vacuum filtration and the paper is washed once with enough distilled water to bring the final volume to about 50 ml.

To this is added 5 ml. of a 50 per cent solution of trichloracetic acid. Following stirring, the protein precipitate is allowed to settle overnight. If precipitation is carried out in volumes smaller than 50 ml., the precipitate tends to form aggregates large enough

to give lumpiness to the film to be formed therefrom; reliable measurements of radioactivity demand a very finely divided precipitate at this step.

After settling, the supernatant is poured off and the precipitate collected on a weighed piece of Whatman No. 540 filter paper in a stainless steel filter, Tracerlab E8A. It is sucked just dry, and washed with 2 ml. of 1 per cent trichloracetic acid. The filter paper bearing the precipitate is then transferred to a brass planchette with a brass ring to hold the filter paper flat (Fig. 2). A single drop of an aqueous solution, 2 per cent urea, 7 per cent formaldehyde, is allowed to spread over the precipitate. The planchette

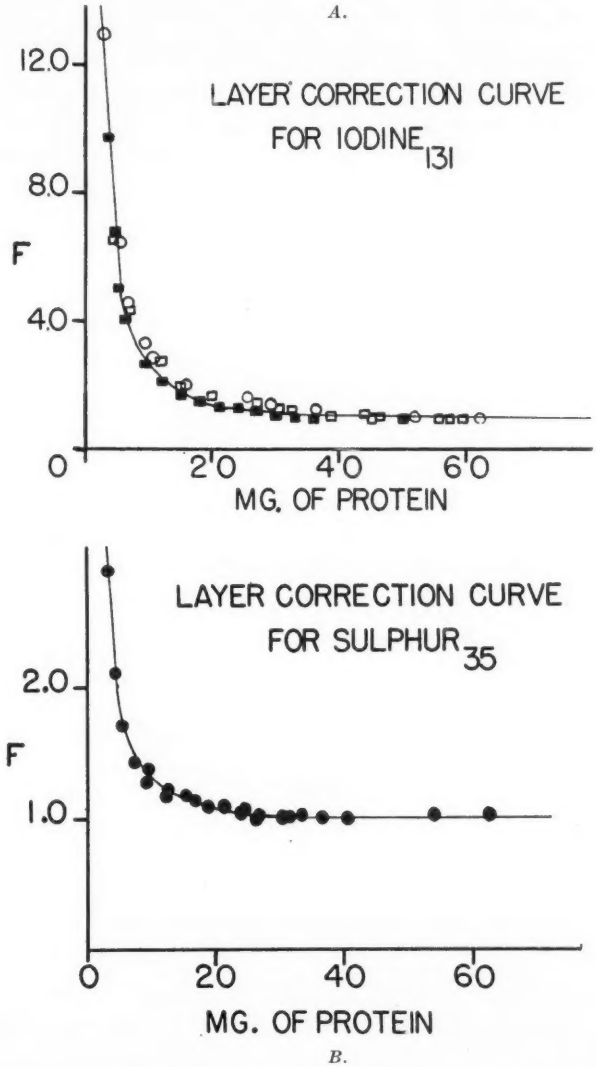



Fig. 4.—*A* and *B*, Self-absorption correction curves in terms of milligrams of protein in film of radius for (*A*) albumin $I_{131}$ and (*B*) serum proteins $S_{35}$. The ordinate here stands in reciprocal relationship to the ordinate of the prototype correction curve illustrated by Voigt[24] in his Fig. 11.

is then transferred to an oven at 100° C. and dried for twenty minutes. The filter paper is then securely clamped between two plexiglass rings (Fig. 2) and further oven dried at 100° C. to constant weight. The plexiglass rings are needed to keep the filter paper from curling during drying. The urea-formaldehyde solution gives a film smooth and glassy on its

upper surface and firmly adherent to the paper below. Reproducibility in measurement of radioactivity is closely related to uniformity in film formation. Cracked films, or films with bubbles or unevenness introduce sufficient technical error into results as to make interpretation difficult.

*Determination of Completeness of Elution.*—We initially checked the completeness of elution by the construction of distance concentration curves for the strips of paper and the comparison of this with the schlieren diagram of the serum by free electrophoresis. In this construction, the paper has been serially sectioned at right angles to the direction of migration, and the protein in each strip (3 mm. wide) has been eluted after the fashion of Kunkel and Tiselius.[9] A plot of the optical densities against the order of strips gives a distance concentration curve grossly identical with that obtained by free electrophoresis (Fig. 3).

Because of difficulties arising from differing affinities of the components for dye,* and because the checks were never much closer than the limits indicated in Fig. 3, more recently we have compared directly the weight of the component eluted from the paper with the weight predicted from known protein concentrations and distributions by free electrophoresis. The results of three experiments, given in Table II, are representative of the closer checks thus achieved; the few results outside this range have been discarded from subsequent consideration on the basis of incomplete extraction.

TABLE II. RECOVERY OF PROTEIN BY EXTRACTION* FOLLOWING PAPER ELECTROPHORESIS†
(DRY WEIGHTS IN MG. PROTEIN)

| PATIENT | ALBUMINS | | $\alpha_1$-$\alpha_2$-$\beta$ GLOBULINS | | $\gamma$ GLOBULINS | |
|---|---|---|---|---|---|---|
| | EXPECTED | FOUND | EXPECTED | FOUND | EXPECTED | FOUND |
| R. Y. | 30.0 | 31.6 | 17.9 | 18.3 | 15.1 | 17.5 |
| G. K. | 30.4 | 30.2 | 18.3 | 16.4 | 10.7 | 10.2 |
| T. P. | 27.3 | 27.8 | 19.9 | 19.7 | 22.5 | 23.4 |

*The expected yields are calculated from the volume of serum subjected to paper electrophoresis, its known total protein concentration, and its distribution of electrophoretic components as shown by analysis of the schlieren diagram of free electrophoresis.

†The results are consistent within the limits of error of the methods with complete extraction.

*Measurement of Radioactivity.*—This is carried out in a windowless flow counter, Tracerlab SC 16 with 100 scaler. All counts have been made to 4,000 counts or thirty minutes. These are corrected for layer thickness in terms of self-absorption curves determined in the conventional fashion both for $S_{35}$ labeled plasma and $I_{131}$ labeled albumin and shown in Fig. 4, *A* and *B*. Results are then corrected for background and radioactive decay.

## RESULTS

*The Problem of "Tailing."*—The electrophoretic components of plasma are notoriously gross mixtures united only by closely similar mobilities asymmetrically distributed about six means at pH 8.6. That further grossness might make apparent turnover times of such mixtures difficult for interpretation was suggested at the beginning of our studies by this finding: when $I_{131}$ labeled albumin† was injected into a normal and two cirrhotics intravenously, the radioactivity was found thus distributed among the paper electrophoresis fractions of withdrawn serum:

| Albumins | $\alpha_1$-$\alpha_2$-globulins | $\beta$-globulins | $\gamma$-globulins |
|---|---|---|---|
| 82 per cent | 15 per cent | 2 per cent | 1 per cent |

*The unpublished work of Roboz, Hess, and Temple[23] on artificial mixtures of albumins and gamma globulins has cast some doubt on correction factors[9] based on the existence of differing affinities.

†These albumin preparations were obtained from Abbott Laboratories, North Chicago, for the studies begun by Schoenberger and co-workers[17] in 1951. In our in vitro work, albumins were further dialyzed to remove any traces of unbound $I_{131}$ remaining after the older Abbott processing. (Cf findings of Berson and co-workers.[12])

Electrophoresis of these $I_{131}$ labeled albumins, both in isolation and added to dialyzed electrophoretically homogeneous unlabeled normal serum albumin, gave no comparable "tailing" either by paper or free electrophoresis. Over 98 per cent of activity was localized to the albumin position.

"Tailing" proved independent of time and of method of addition to sera. Identical distributions of "tailed" activity (never greater than 20 per cent) were found both by paper and free electrophoresis under these conditions:* (a) in vitro addition at 5° C.; immediate electrophoresis; (b) in vitro addition; electrophoresis after standing twenty-four hours at 5° C. and 37° C.; (c) intravenous injection; sera withdrawn after one hour, twenty-four hours, and two weeks, and subjected to immediate electrophoresis and electrophoresis after standing twenty-four hours at 5° C. and 37° C.

This "tailing" raised the question of existence of complexed albumins in the slower components either under normal conditions or as a result of fractionation and labeling procedures. Therefore, we prepared electrophorectically homogeneous albumins and gamma globulins from human sera whose proteins had been labeled in vivo by oral administration of $S_{35}$ containing yeast.† Method $10^{5, 6}$ and (for albumins) the rework step of Method $5^{18}$ were used.

Addition of albumins so separated to normal sera gave strikingly less "tailing" (rarely in excess of 5 per cent). When gamma globulins so prepared were added, radioactivity was localized to those segments of paper corresponding to the radioactivity of the globulins in isolation.

In the last three months, we have repeated the $I_{131}$ labeled albumin experiments with the most recent Abbott Laboratory preparations.‡ For these (whose preparative and other specifications differ from those applicable to the $I_{131}$ albumins first studied) "tailing" has been less than for the in vivo $S_{35}$ labeled albumins separated in our own laboratory (rarely greater than 2 per cent).

Thus while complexing of albumins in slower electrophoretic components does not appear to complicate the appraisal of "apparent turnover times" when labeled by in vivo synthesis, we believe that our initial "tailing" findings reinforce the opinion of Berson and co-workers[12] that proteins labeled outside the body must be regarded with considerable circumspection, both with regard to changes induced thereby in metabolic pathways, and in immediate interactions with other proteins.

*Clinical Studies.*—Twelve patients (in addition to those in the "tailing" experiments) have had studies of the persistence of radioactive labels in plasma protein fractions. Of the five patients believed to be normal at the initiation of studies, two are still so believed at time of writing; one developed evidence of infectious mononucleosis between $S_{35}$ and $I_{131}$ administration; one developed a cerebral thrombosis (presumably arteriosclerotic) over a year after completion of observations; one has evidence of early, uncomplicated, essential hypertension.

*Because of difficulty in obtaining alpha$_1$-alpha$_2$ globulin fractions free of beta and gamma globulins in the Tiselius cell, free electrophoresis gave results in qualitative rather than quantitative confirmation of the "tailing" seen in paper electrophoresis.

†Obtained from Abbott Laboratories, North Chicago, Illinois.

‡We are indebted to Dr. Donalee Tabern of Abbott Laboratories for this material and for many helpful discussions during the course of the work.

Four patients were studied in severe cardiac decompensation of varied etiology. Three were selected by reason of absence of gross abnormalities in plasma protein distribution measured by free electrophoresis; one by reason of hypoalbuminemia associated with chronic adhesive pericarditis. (All of these patients had detailed studies of cardiac dynamics by catheter, together with liver function studies, in collaboration with Drs. Robert Grissom, Luke Pascale, and Angelo Creticos, which will be reported separately.)

Three other patients were studied: one with amyloid nephrosis, one with disseminated lupus erythematosis inactive for over one year, and one with a syndrome associated with absence of gamma globulins.*

Eleven patients (in addition to those who furnished sera for "tailing" studies) received $S_{35}$ labeled yeast† orally. Serial sera were withdrawn over periods varying between three and eleven weeks. Of these, three patients received $I_{131}$ labeled albumin following the $S_{35}$ study; serial sera over an additional two to three weeks were collected, fractionated, and counted. One patient received $I_{131}$ intravenously before receiving $S_{35}$; completion of the latter study was rendered impossible by clinical developments.

All patients received oral supplements of 4 Gm. methionine and 2 Gm. cystine during $S_{35}$ studies except as noted hereafter. Patients given $I_{131}$ albumin received Lugol's solution 10 drops three times a day from injection to completion of experiments.

Appropriately corrected radioactivity values for the following fractions: Albumins, a mixture of alpha-1, alpha-2, and beta globulins (globulins of intermediate mobility) and gamma globulins were plotted semilogarithmically against time.

*The Form of the Plots.*—In Fig. 5 (for description, see legend) are plotted the data for a typical experiment (Patient R. R., Table III). Measurements were carried out over two thirty-five-day periods following $S_{35}$ ingestion and $I_{131}$ labeled albumin injection, respectively. A similar seventy-seven-day experiment (Patient R. C., Table IV), in which $S_{35}$ alone was given, is plotted in Fig. 6.

While the $S_{35}$ points after the third day (Fig. 5) lie in about the indicated lines with no greater scatter than is seen for the $I_{131}$ points, the considerable scatter of the initial points does not permit reliable graphic resolution of any plot into the sum of more than two exponential regression rates.‡

The faster exponential is presumed to reflect equilibration of the newly synthesized fraction with the extravascular pool or pools. Construction of the faster exponentials for the initial experimental points of Fig. 7 (see legend) is

*We are indebted to Drs. Frank Trobaugh and Harry Alexander of Washington University, St. Louis, for making available this patient for study.

†Obtained from Abbott Laboratories, North Chicago. Dosage was 500 microcuries in patients believed to have a prognosis longer than two years, 1.0 millicurie in patients with a graver prognosis. All the latter are now dead; diagnoses were confirmed at autopsy in all but one, where permission was not obtainable. Dosage of $I^{131}$ labeled albumin was comparable to that reported by other workers in this department,[17] intravenously administered.

‡The mathematical analysis of these and other relevant plots is given by Berson and co-workers.[12]

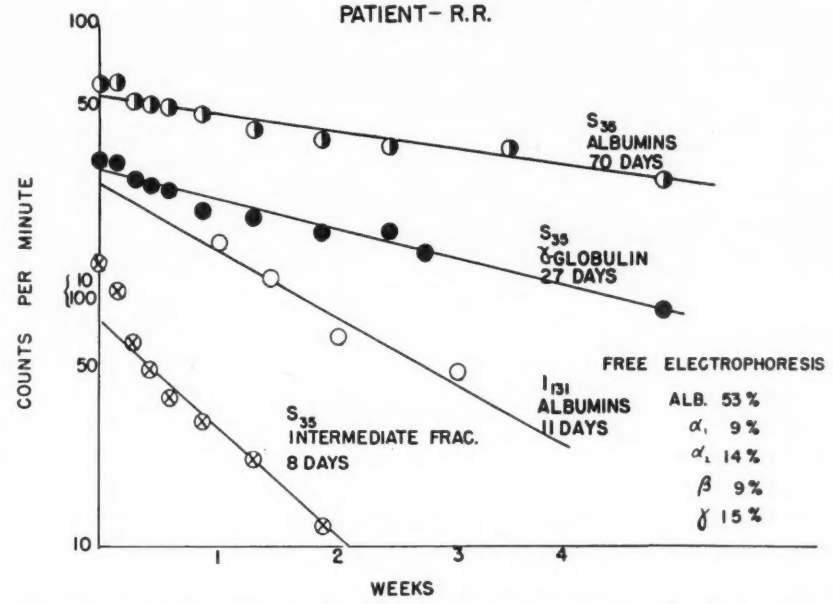



Fig. 5.—Semilogarithmic plot of counts per minute vs. time for a typical experiment (Patient R.R.). The corrected counts per minute above background (ordinate) apply only to the $S_{35}$ labeled fractions. The points for days 1-3 on the $I_{131}$ albumin plot were lost due to laboratory accident. The remainder lie in the range 2,200 to 100 over background. The comparatively normal free electrophoretic distribution of proteins is indicated by the data in the lower right hand corner of the Fig. If the line for $I_{131}$ albumins was extended to this corner, it would pass through the thirty-five-day point.

The number of days indicated for each line is the "apparent turnover time" ($t_{1/2}/1n2$) estimated from the final slope.

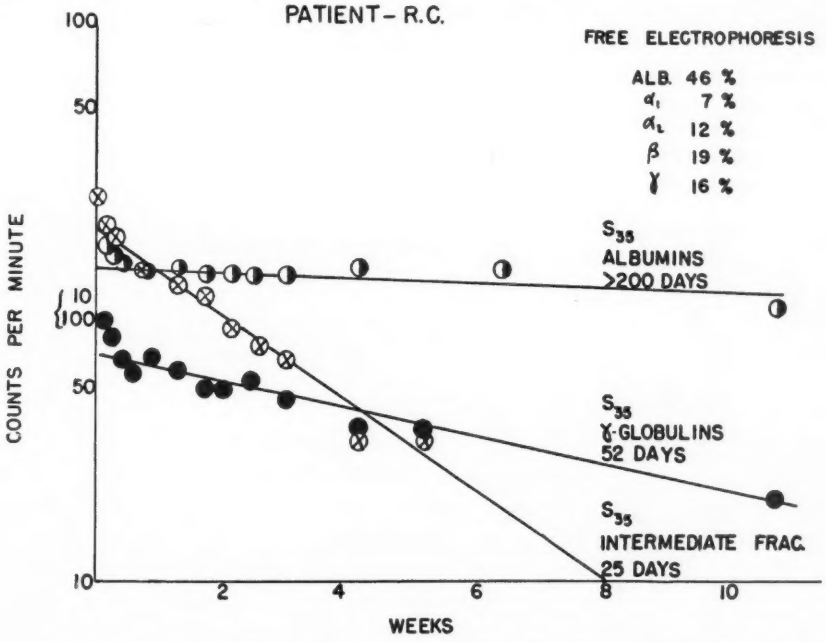



Fig. 6.—A plot (similar to Fig. 5) of a seventy-seven-day experiment following ingestion of $S_{35}$. Death of the patient precluded a study following intravenous injection of $I_{131}$ labeled albumin. The comparatively normal free electrophoretic distribution of protein components, despite prolongations of "apparent turnover times," is indicated by the data in the upper right hand corner.

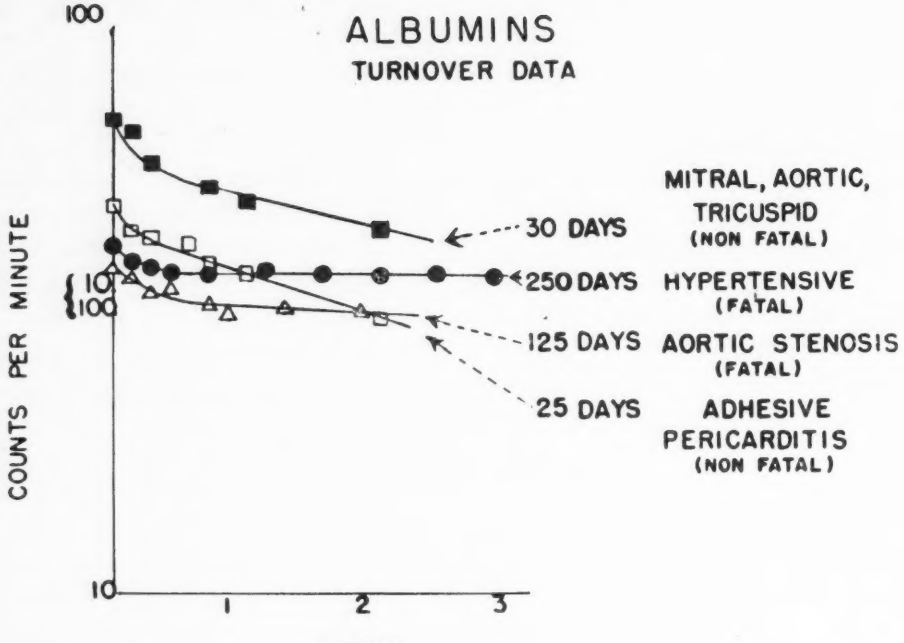



Fig. 7.—Plots (similar to those of Figs. 5 and 6) giving the experimental data on the albumin fraction for periods between two and three weeks following $S_{35}$ ingestion for patients R.Y., R.C., L.K., and L.S. (Table IV).

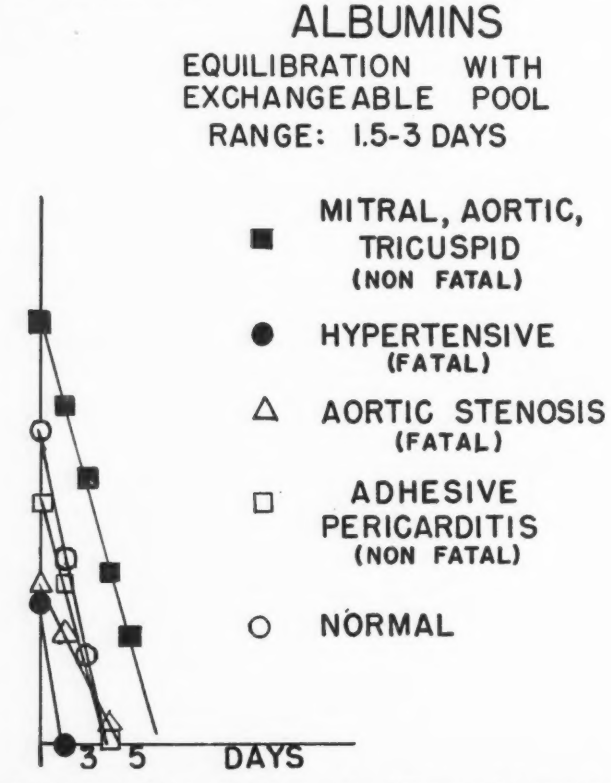



Fig. 8.—Semilogarithmic plot against time of the differences between the smoothed curves of Fig. 7 and extrapolation of the final slope of these curves to zero time. The rate constant of the "faster" exponential regression[12] is characterized by the slopes of these plots. A similar plot for a normal patient is included for comparison.

illustrated in Fig. 8 (see legend) and indicates that the equilibration of newly synthesized albumins with the extravascular metabolic pool in severe congestive failure is not grossly slowed as compared to a normal subject.

Similar constructions for all fractions studied have indicated that mixing is virtually complete in three to four days. Whether there exist extravascular pools characterized by faster equilibration cannot be ascertained from our data. The slower exponential is presumed to reflect metabolic degradation of the labeled fraction. For this, an estimate of the "apparent turnover time" ($t_{1/2}/1n2$) can be made (with reliability considered below) from the final slopes of the plots as illustrated in Figs. 5-7.

The "apparent turnover times" thus obtained from these plots have shown: (a) fairly consistent differences in the same subject between the albumins synthesized following ingestion of $S_{35}$ and albumins $I_{131}$ labeled and intravenously injected (see the topmost, and next to bottom slopes in Fig. 5); (b) fairly consistent differences in the same subject between the $S_{35}$ labeled albumins and gamma globulins on one hand, and the globulins of intermediate mobility on the other (for example compare top, next to top, and bottom lines, Fig. 5, with corresponding lines, Fig. 6); (c) far less consistent differences for each $S_{35}$ labeled fraction when measured in several subjects, normal and abnormal. Let us consider these differences in sequence.

*Comparison of Apparent Turnover Times of Albumins Labeled by $I_{131}$ and $S_{35}$.*—The complete data for three initially normal patients who received both isotopic labels are given in Table III.

TABLE III. DATA ON THREE SUBJECTS WHO RECEIVED BOTH $I_{131}$ ALBUMIN INTRAVENOUSLY AND $S_{35}$ ORALLY

| | DISTRIBUTION OF FREE ELECTROPHORETIC COMPONENTS (%) | | | | | APPARENT TURNOVER TIMES (DAYS) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | ALBUMINS | | INTERMEDIATE GLOBULINS | | GAMMA GLOBULINS |
| PATIENT | ALBUMINS | ALPHA-1 | ALPHA-2 | BETA | GAMMA | $S_{35}$ | $I_{131}$ | $S_{35}$ | ($I_{131}$) | $S_{35}$ |
| L. M. | 50 | 5 | 15 | 16 | 14 | 68 | 19 | 19 | (18*) | 65 |
| L. D. | 53 | 4 | 8 | 18 | 17 | 37 | 14 | 15 | —† | 87 |
| R. R. | 53 | 9 | 14 | 9 | 15 | 70 | 11 | 8 | (10*) | 27 |

*This figure doubtless represents "tailing" of $I_{131}$ labeled albumin. For discussion, see text.

†No activity in this position indicates no "tailing."

With respect to the $I_{131}$ label, the fact that the apparent turnover times of the "tailing" albumins in electrophoretic fraction of intermediate mobility in subjects L.M. and R.R. differ by only a day from the albumins migrating with characteristic mobility, gives an internal check on the reproducibility of the techniques which is probably too good.

As a further check on the range of values thus obtained with the $I_{131}$ label, we have studied one additional subject with heavy albuminuria and two without albuminuria with these results:

| Diagnosis | Apparent Turnover Time (Albumin $I_{131}$) |
|---|---|
| Amyloid nephrosis | 11 days |
| Normal subject | 14 days |
| Laennec's cirrhosis | 15 days |

(These subjects did not receive $S_{35}$)

From Table III, two facts are immediately apparent: First, these apparent turnover times of $I_{131}$ labeled albumins are comparable to the results of other workers with $I_{131}$ labeled albumins (Table I) with this important exception: the results of Berson's group with albumins "free of rapidly degraded components."* Second, the apparent turnover times of autosynthesized $S_{35}$ albumins are at least twice as long as the times of $I_{131}$ albumins injected in the same patients. While the thirty-seven days for subject L. D. is close to Berson's results for $I_{131}$ albumins "free of rapidly degraded components" in decompensated hepatic cirrhosis, there was no clinical or laboratory evidence of this condition. Subjects L. M. and R. R. show even greater prolongation. Why these discrepancies? Could they be due to uncertainties of the graphical estimates made from the crude data?

We believe they are too large to be thus accounted for. In our early work we carried out duplicate determinations in one normal subject. At that time, we used an experimental period comparable to that customary for the $I_{131}$ label (about two weeks) and obtained these estimates:†

| *Albumins* | *Gamma Globulins* |
|---|---|
| twenty-four days | > one hundred days |
| thirty-two days | > one hundred days |

Though the agreement between the slopes for the two albumin lines was not as close as the existing $I_{131}$ data had led us to anticipate, and though the experimental period was too short to permit the valid assignment of a number between 100 and 00 to characterize the slopes of two puzzlingly flat gamma globulin plots,‡ the obvious difference in the slope between albumins and gamma globulins in each experiment provided enough of an inkling of range of reproducibility to encourage us to continue measurements with the more extended observation periods illustrated in Figs. 5 and 6.

Formal statistical treatment is inapposite at this stage of our work. Let us substitute, pro tempore, the eye. In Fig. 5 (five-week experiment) if, for a hypothetical fraction, a line were drawn of slope corresponding to fifty days between the topmost ($S_{35}$ albumins, seventy days) and the next (gamma globulins, twenty-seven days) lines, a glance at the scatter of experimental points indicates the uncertainty in stating that the apparent turnover time of such a hypothetical fraction might be significantly different from either measured fraction. A glance likewise indicates the lack of difficulty in stating that the topmost fractions do differ significantly from those represented by the two bottom lines. The differences in slopes of the nine-week experiment of Fig. 6 are even more striking.

Could the prolongation of apparent turnover time of $S_{35}$ autosynthesized albumins be due to reutilization of the isotope in synthesis during the experimental period?

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*As indicated in the methods sections, our most recent studies were with $I_{131}$ albumin which showed no "tailing" in electrophoresis either by our hands or by other workers (personal communication of Dr. Donalee Tabern of Abbott Laboratories). However, we do not yet have the facilities for the type of ultracentrifugal analyses carried out by Berson's group.

†The measurements on globulins of intermediate mobility in this subject were discarded for technical reasons.

‡Dr. W. Volwiler, of the University of Washington, Seattle, has personally advised us that with the $S_{35}$ label and different methods of fractionation and preparation for radioactivity estimates, he has encountered negligible rates of disappearance of radioactivity from the gamma globulins.

The feeding of supplementary unlabeled S containing amino acids is aimed to prevent this. Madden and Gould[16] found approximately the same turnover rates for fibrinogen in three human subjects. Of these, two received such supplements, one did not. We attempted to extend their findings thus: In Patient R. C. the supplements were withheld for the first month. The experimental points after their administration indicated no significant change in slopes for the three fractions under study.*

Unfortunately, none of these considerations rule out reutilization in some degree.†

TABLE IV. DATA ON SIX CARDIAC PATIENTS WITH AND WITHOUT CONGESTIVE FAILURE

| PATIENT AND DIAGNOSIS | CONGESTIVE FAILURE | DISTRIBUTION OF FREE ELECTROPHORETIC COMPONENTS (PER CENT)* | | | | | APPARENT TURNOVER TIMES | | |
|---|---|---|---|---|---|---|---|---|---|
| | | ALBUMINS | $\alpha_1$ | $\alpha_2$ | $\beta$ | $\gamma$ | ALB. | $\alpha_1\alpha_2\beta$ | $\gamma$ |
| J. M.‡ Hypertension; Recent healed | Absent | 50 | 4 | 13 | 16 | 17 | 77 | —† | —† |
| T. P. Cardiac Infarction | Absent | 39 | 5 | 8 | 16 | 32 | 34 | 15 | 41 |
| R. Y. Aortic stenosis and Mitral insufficiency; Tricuspid insufficiency | Chronic Severe | 48 | 5 | 7 | 15 | 25 | 30 | 18 | 52 |
| L. S. Constrictive and adhesive pericarditis | Chronic Severe | —* | —* | —* | —* | —* | 24 | 8 | 88 |
| L. K. Calcific aortic stenosis | Severe Fatal | 51 | 5 | 10 | 16 | 18 | 128 | 14 | 113 |
| R. C. Hypertension; Nitrogen retention | Severe Fatal | 46 | 7 | 12 | 19 | 16 | >200 | 25 | 52 |

*All patients save L. S. had total serum proteins within the normal range. L. S. had consistent hypoproteinemia (4.0-4.5 Gm. per cent) with an albumin level of about 2 Gm. per cent. (Free electrophoresis is not available on L. S.)

†Estimates discarded for technical reasons.

‡This patient had suffered from disseminated lupus erythematosis and has been previously reported on by one of us.[20] There was no evidence of activity at time of study.

Could the differences between apparent albumin turnover time of the $S_{35}$ and $I_{131}$ labels be due to the fact that those albumin molecules which are readily iodinated contain very little S. Those molecules which are high in S cannot be readily iodinated and these two classes of albumins have different intrinsic turnover times. Nothing in our data rules this possibility in or out.

*Comparison of Apparent Turnover Times of the $S_{35}$ Labeled Fractions in the Same Subjects.*—In Table IV are given the apparent turnover times of the three fractions and other relevant information for six patients with heart disease (two without and four with congestive failure).

*In Fig. 6 certain experimental points in weeks 7-8, omitted in lettering the figure, show no more than the usual scatter about the drawn lines.

†The oral administration of $S_{35}$ does not permit the use of a "distribution curve" (a plot against time of $\frac{\text{counts per minute per unit volume of plasma}}{\text{per cent of administered isotope retained}}$ to settle this matter. The uncertainty of gastrointestinal absorption leads to uncertainty in the denominator of this fraction, that is, one does not know the fraction of the oral isotope which becomes incorporated in plasma proteins at the start of the experiment. For this reason also, one cannot calculate the magnitudes of exchangeable extravascular pools, as can be done when a labeled protein is administered intravenously.

From both Table III and Table IV two findings stand out: First, in all subjects, the apparent turnover time of the fraction of intermediate mobility is either about one-half or less than one-half the values for the albumins or gamma globulins. This holds as well for patients in Table III as Table IV, and also holds (with respect to albumins) for the intermediate fraction of the patient with absent gamma globulins, whose data follow:

| *Free Electrophoresis* (per cent) | | | | | *Apparent Turnover Times* (Days) | | |
|---|---|---|---|---|---|---|---|
| Albumins | $\alpha_1$ | $\alpha_2$ | $\beta$ | $\gamma$ | Albumins | Globulins of Intermediate Mobility | $\gamma$ |
| 54 | 13 | 19 | 14 | 0 | 35 | 8 | — |

Second, there seems no consistent relationship between apparent turnover times of albumins and gamma globulins. Thus, in Table III, L. M. has apparent turnover times for both albumins and gamma globulins which are essentially identical and about twice the normal values previously reported with $S_{35}$ (see Table I). L. D.'s albumins time is half that of his gamma globulins and only slightly above the previously reported normal value. R. R.'s gamma globulins time is half that of the albumins and likewise close to the previously reported normal value.

TABLE V. DAY ONE COMPARISON TO ALBUMINS OF INITIAL RADIOACTIVITIES OF ALPHA$_1$-ALPHA$_2$-BETA GLOBULINS AND GAMMA GLOBULINS OF SUBJECTS OF TABLES III AND IV

| SUBJECT | $\alpha_1$-$\alpha_2$-BETA GLOBULINS | GAMMA GLOBULINS |
|---|---|---|
| L. M. | 89 per cent | 36 per cent |
| L. D. | 90 per cent | 27 per cent |
| R. R. | 138 per cent | 54 per cent |
| J. M. | —* | 43 per cent† |
| T. P. | 105 per cent | 54 per cent |
| R. Y. | 22 per cent | 7 per cent |
| L. S. | 67 per cent | 27 per cent |
| L. K. | 143 per cent | 43 per cent |
| R. C. | 133 per cent | 50 per cent |
| Mean Values | 98 per cent | 38 per cent |

*Data discarded for technical reasons.

†Though technical problems with data in the second week after $S_{35}$ administration did not permit a reliable estimate of the final slope of the plot, the figure here given derives from an intercept based on the first week's data.

We must now ask a question analogous to that in the last section: Can different rates of reutilization of the isotope in synthesis of the fractions be related to these different apparent turnover times? The implications of different isotope concentrations in different metabolic pools does not make this an easily accepted explanation. The only relevant data we have are as follows:

In Table V are given, for the subjects of Tables III and IV, comparisons of the initial radioactivities of the globulins of intermediate mobility and of the gamma globulins with the albumins fraction. Expressed as percentage of the initial albumins radioactivity, the values for these comparisons come from extrapolation of the final slopes of the curves (as illustrated in Fig. 5) to zero time. This gives an estimate of the initial radioactivity of the labeled fraction as if already in equilibrium distribution with extravascular protein pool or pools.

Though there is considerable variation between subjects, the averages show values for albumins and intermediate fractions which are about equal and over

twice the gamma globulins value. The most striking individual variation is subject R. Y., whose initial albumins radioactivity was four times that of his intermediate fraction and over ten times that of his gamma globulins. In no subject was the initial gamma globulins radioactivity significantly greater than half that of either other fraction.

Brand and associates'[18] analyses of early ethanol-water fractionations[19] of pooled normal plasma indicate that gamma globulins have by weight about half the protein S as do albumins. (Analyses for a gross $alpha_1$-$alpha_2$-beta mixture comparable to the intermediate fraction here studied are not available). The fact that the intermediate fraction radioactivity persists for a far shorter period in the plasma than that of the albumins despite not too different initial levels; the fact that persistence of gamma globulin radioactivity is more comparable to that of albumins despite a consistently smaller initial level; and the fact that initial levels of the latter fractions can be better correlated with protein S content than with apparent turnover times suggest that different isotope reutilization rates are probably not the major factor in different apparent turnover times. Beyond this suggestion our data cannot take us.

*Comparison of Apparent Turnover Times of Individual Fractions Between Subjects.*—Turning first to the albumins fraction, of the two shortest apparent turnover times (L. S., 24 days, R. Y., 30 days) both were associated with severe congestive failure and bromsulphthalein* retention of 15 per cent at forty-five minutes; of these, only L. S. showed significant lowering of serum albumin level. The next shortest times (T. P., thirty-four days, L. D., thirty-seven days) were found in subjects without congestive failure, bromsulphthalein retention, or hypoalbuminemia. The longest (G. K., 128 days, R. C., 200 days) were associated with congestive failure fatal shortly after the experimental periods, bromsulphthalein retentions of 17 per cent and 30 per cent, respectively, in forty-five minutes. Of these one (R. C.) had nitrogen retention in part due to severe nephrosclerosis;† there was miminal nitrogen retention and nephrosclerosis† in subject G. K.

No more promising leads for future clinical correlations have emerged from the gamma globulin data than from the data on albumins. The subject with the longest albumins time had a gamma globulins time (fifty-two days) only slightly greater than the previously reported normal with $S_{35}$. The next to longest gamma globulins time (eighty-eight days) was associated with one of the shortest albumins times (twenty-four days).

For neither albumins nor gamma globulins fraction is there any obvious correlation between the blood level of the component by free electrophoresis and the apparent turnover times by paper electrophoresis.

For the fraction of intermediate mobility, one can do little more than point out that the subject with the longest albumins time had the longest $alpha_1$-$alpha_2$-beta time (twenty-four days); of these three subjects with shortest $alpha_1$-$alpha_2$-beta times (eight days), one (L. S.) was in chronic severe congestive failure and

*Dose: 5 mg. per kilogram body weight.
†Autopsy finding.

had the next to longest gamma globulins time (eighty-eight days), one was a normal subject (R. R.) whose gamma globulins time (twenty-seven days) was in the previously reported normal range, and one suffered from the multiple infectious episodes associated with absence of circulating gamma globulins. No radioactivity appeared on the gamma position on the paper; turnover was unmeasurable.

### APPRAISAL OF RESULTS

These are results of the crudest sort. In the attempt to standardize techniques for measurement of plasma protein turnover sufficiently free of laborious detail to permit multiple clinical studies, a considerable amount of labor has gone into the achievement of the limited reproducibility here reported.

The three fractions studied with $S_{35}$ are obviously and extraordinarily crude. If few preparations of human albumin, homogeneous by electrophoresis and in the ultracentrifuge, can meet the thermodynamic solubility criteria for identity of a molecular species, we need not pause to discuss "purity" of paper electrophoretic fractions, even in the absence of "tailing." For this reason we have avoided the term "biologic half life" with its covert implication of molecular species homogeneity. Though the numbers given for "apparent turnover times" emerge from the same graphical analysis of data that is customarily used for "biologic half lives," the former term emphasizes that within our experimental limits a group of molecular species with very considerable differences in intrinsic biologic half lives could, if lumped in one of our crude fractions, yield an apparently linear plot which would both fail to reveal either the inhomogeneity or the degree of individual differences.

Part of the price of reproducibility has been this crudeness. Given the $S_{35}$ dosage limits in effect when we started this study, and the increased uncertainty of the self-absorption radioactivity correction (Fig. 4*B*) for eluted fractions less than 10 mg. in weight, we have patently sacrificed more detailed fractionation for weight in studying the alpha$_1$-alpha$_2$-beta globulins as a mixture. And again, once having learned to extract from filter paper in a complete and reproducible fashion (Table II), we were loathe to shift to supportive media allowing larger fractions (for example, starch) until we had accumulated some data.

Knowing in advance this crudeness, we realized that gross differences in apparent turnover times alone would have significance. The differences we anticipated were in the direction of shortness. Thus, for example, we wondered if the apparent turnover of the gamma globulins in diseases of presumed hyperimmune etiology (in which circulating antibodies against tissue components have been notoriously difficult to demonstrate[21]) might prove significantly faster than in the normal, due to hypothecated excesses of fixed tissue antigen which might remove some part of this fraction as fast as it is synthesized.

We were led to include cardiacs as readily available hospitalized controls. When we first encountered gross differences in apparent turnover times in the direction of increased length, we were not only surprised but we felt the com-

parison of L. K. and R. C. with the far shorter times encountered in our first short term studies of the normal by $S_{35}$ and $I_{131}$ might indicate "radical shifts in protein dynamic equilibria in the presence of cardiac decompensation."[22] The subsequent longer term studies of Table IV make us look with some doubt on the early short term $S_{35}$ studies, and make the prolongations of apparent turnover time much more difficult of interpretation. Indeed we do not feel any sure metabolic interpretation with reference to health or disease can be given these results at this time.

We present them because we believe the difficulties encountered in our first two years of work will be of interest to the increasing number of workers in the field of the intermediary metabolism of plasma proteins. We plan to continue our studies in presumably normal and uremic subjects, using higher doses of supplementary S containing amino acids, in a further study of the problem of reutilization. Also, with increased dosage of $S_{35}$ now permitted, we plan to supplement crude paper electrophoretic fractions with fractions more specific in known biologic function. We do not feel that large-scale study of disease states is warranted until the questions of methodology and interpretation of the results here presented can be better dealt with than at present.

## SUMMARY

Employing orally administered $S_{35}$ as a label, we have studied in human subjects apparent rates of turnover of these three crude plasma protein fractions quantitatively extracted following filter paper electrophoresis: albumins, a mixture of $alpha_1$-$alpha_2$-beta globulins, and gamma globulins.

Reproducibility of results with existing $S_{35}$ dosage limits has in part dictated the crudeness of fractionation and has necessitated great meticulousness in techniques of both extraction and film preparation for radioactivity measurements. Avoidance of further crudeness has required special care with conditions of paper electrophoresis.

Our initial finding of some 20 per cent of radioactivity of $I_{131}$ labeled albumins added to sera in the $alpha_1$-$alpha_2$ positions on the paper has been proved in part dependent on conditions of electrophoresis, in part on the added albumins preparation. Such "tailing" has rarely exceeded 5 per cent with $S_{35}$ labeled albumins prepared by ethanol water fractionation in our laboratory or with recent commercially available $I_{131}$ albumin lots.

Turnover results are reported as "apparent turnover times" to avoid any implication of a number characteristic of the biologic half life of any single molecular species.

For the albumins fraction, apparent turnover times have been either in the same range or longer than results others have reported with the $S_{35}$ or the $N_{15}$ label. In three subjects where we have employed both orally ingested $S_{35}$ and intravenously injected $I_{131}$ labeled albumins, $I_{131}$ results have been in the range reported by others; $S_{35}$ results (in "die away" experiments between three and six weeks in duration) have yielded apparent turnover times between twice and four times as long as with $I_{131}$. The longest apparent turnover time found for albumins exceeded 200 days in an experiment of nine weeks' duration.

Similar prolongations have been observed for the gamma globulins fraction. (The longest was 113 days.)

In all eleven subjects studied with $S_{35}$, the apparent turnover times of the alpha$_1$-alpha$_2$-beta globulins fraction was significantly less than either of the other two fractions. (The shortest was eight days, the longest twenty-four days).

Though the subjects studied included normals and several pathologic conditions, we do not believe any sure metabolic interpretations can be given to our results at this time. We believe they are of significance chiefly in terms of the methodologic and interpretive problems which they raise for studies of this sort. They are discussed chiefly from this standpoint.

We are indebted to Miss Ruth Sights and Mrs. Margaret Chakinis for technical assistance in the development and carrying out of these experiments.

We are grateful to the Otho S. A. Sprague Memorial Institute for funds to initiate this work, and to the United States Public Health Service for a grant to support its extension.

The senior author is indebted to the Dr. Leonard H. and Louis D. Weissman Medical Research Foundation for the backing of the studies of plasma proteins as related to disease mechanisms at the Hektoen Institute for Medical Research of Cook County Hospital.

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# PRELIMINARY OBSERVATIONS ON THE EFFECT OF LEVALLORPHAN ON RESPIRATORY DEPRESSION AND ANALGESIA OF LEVORPHAN IN MAN

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IT HAS been demonstrated that levallorphan tartrate will antagonize the respiratory depression and the analgesia that is produced by administration of potent analgetics in animals[1, 2] and respiratory depression in man.[3, 4] Fromherz and Pellmont[1] have suggested that in animals respiratory depression is antagonized with smaller doses than that required to inhibit the analgesia. It is of considerable clinical interest to determine whether such a spread of dosages holds true in man. That is, is it possible to combat the respiratory depression of potent analgetics without abolishing their analgetic activity? This study was undertaken as a preliminary step in investigation of this possibility.

## METHODS

For the analgetic studies the Wolff-Hardy-Goodell technique was employed using five trained subjects. Graded doses of levallorphan tartrate were given subcutaneously before, simultaneously, and after, a fixed dose of levorphan tartrate.* Both placebos and the drugs were employed in a randomized fashion. Readings of the threshold rises were made at thirty-minute intervals. Each subject was observed for a period of 150 minutes following the injection. Maximum rises of threshold were used for calculation purposes.

A subcutaneous dose of 1 mg. levorphan tartrate was used in all instances as the standard dose of analgetic. The following doses of levallorphan tartrate were used against the standard dose of levorphan tartrate: 0.05, 0.1, 0.25, 0.5, 0.75, and 1 mg. The very small doses of levallorphan tartrate which were used initially represented the first trials of this drug in man.

For studies of antagonism of respiratory depression, twenty patients were used. These patients were to have some form of surgery performed and these studies were done as part of the anesthetic procedure. All patients were given levorphan tartrate and levallorphan tartrate in a ratio of 10 units of analgetic to 1 unit of antagonist. The dose of levorphan tartrate used ranged from 5 to 10 mg. In ten patients, the analgetic agent was given first followed by the antagonist at time intervals of from eight to forty-five minutes. In six patients, the antagonist was given first and the analgetic later at time intervals varying from two to fifteen minutes. In four patients, the drugs were given simultaneously. In all cases respiration was observed closely and in some patients minute volume measurements were made. The observations were made when it was felt that maximum change had occurred. All drugs were given by rapid intravenous injection.

## RESULTS

From visual inspection of the analgesia data, the standard dose of levorphan tartrate gave a significant threshold rise above the control readings.

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Received for publication, Feb. 24, 1954.

*Levo Dromoran Tartrate (generic name, levorphan tartrate) and levallorphan tartrate (Ro 1-7700) were supplied for this study by Dr. M. J. Schiffrin, Hoffmann-LaRoche, Inc.

Likewise, it could readily be seen that doses of 0.05 to 0.1 mg. of levallorphan tartrate did not significantly alter the threshold rise produced by the standard control dose of levorphan tartrate. Doses of the antagonist higher than 0.1 mg. were treated statistically by determining the student's "t." Table I gives the P value for each treatment that was submitted to statistical analysis.

TABLE I. STUDIES ON ANALGESIA IN HUMAN VOLUNTEERS

| TREATMENT | AVERAGE MAXIMUM THRESHOLD RISE (PER CENT) | P VALUE |
|---|---|---|
| Saline placebo | 0.88 | — |
| 1 mg. levorphan versus saline placebo | 9.14 | < 0.001 |
| 1 mg. levallorphan + placebo* | 2.20 | < 0.001 |
| 1 mg. levorphan + 0.25 mg. levallorphan simultaneously* | 9.58 | 0.8-0.7 |
| 1 mg. levorphan + 0.5 mg. levallorphan simultaneously* | 6.94 | 0.05-0.1 |
| 1 mg. levorphan + 0.75 mg. levallorphan simultaneously* | 4.3 | 0.01-0.001 |
| 1 mg. levorphan + 1 mg. levallorphan simultaneously* | 2.6 | < 0.001 |
| 0.5 mg. levallorphan, fifteen minutes before 1 mg. levorphan* | 3.0 | < 0.001 |
| 1 mg. levorphan followed in thirty minutes with 1 mg. levallorphan* | 6.1 | 0.5-0.3 |

*The per cent increase in threshold produced by the combinations shown in this table were compared with 1.0 mg. levorphan. Tartrates of levorphan and levallorphan were used throughout.

TABLE II. LEVORPHAN TARTRATE ADMINISTERED PRIOR TO ANTAGONIST

| NO. | LEVORPHAN TARTRATE (MG.) | RESPIRATION AFTER LEVORPHAN | | LEVALLORPHAN TARTRATE | | RESPIRATION AFTER LEVALLORPHAN | |
|---|---|---|---|---|---|---|---|
| | | RATE/ MIN. | MIN. VOL. (ML./MIN.) | MG. | TIME AFTER LEVORPHAN (MIN.) | RATE/ MIN. | MIN. VOL. (ML./MIN.) |
| 1 | 10 | apnea | 0000 | 1.0 | 10 | 16 | |
| 2* | 5 | 11 | 4,400 | 0.5 | 45 | 18 | 7,400 |
| 3 | 9 | 4 | 2,600 | 0.9 | 15 | 12 | 7,500 |
| 4 | 10 | 14 | 5,950 | 1.0 | 25 | 18 | 11,700 |
| 5 | 5 | 9 | | 0.5 | 8 | 14 | |
| 6 | 5 | 8 | | 0.5 | 15 | 18 | |
| 7 | 6 | 10 | | 0.6 | 12 | 20 | |
| 8 | 5 | 12 | | 0.5 | 17 | 18 | |
| 9 | 6 | 6 | | 0.6 | 15 | 12 | |
| 10 | 6 | 5 | | 0.6 | 10 | 16 | |

*Cases 2 through 10 are identical with Patients 32-40 listed in Table II in article now in press.[4]

It appears that dose ratios of 0.75 mg. of antagonist to 1 mg. of analgetic significantly inhibited the threshold rise when given simultaneously (P = 0.01-0.001). A ratio of 0.5 mg. to 1 mg. significantly inhibited the maximum threshold rise when the antagonist was given fifteen minutes before the analgetic (P = < 0.001). Levallorphan tartrate in a 1 to 1 ratio given thirty minutes subsequent to the analgetic produced no statistically significant alteration in threshold (P = 0.5-0.3).

In the respiratory study, the group receiving levorphan tartrate prior to levallorphan tartrate all exhibited some degree of respiratory depression which was antagonized at least partially by levallorphan tartrate (Table II).

In the group receiving the antagonist first, four of the six patients exhibited definite respiratory depression after the analgetic. In these four patients the analgetic was given two to ten minutes after the antagonist. Using similar dosages, two other patients given the analgetic ten and fifteen minutes after the antagonist showed no respiratory depression.

TABLE III. LEVORPHAN TARTRATE ADMINISTERED SIMULTANEOUSLY WITH ANTAGONIST

| NO. | CONTROL (RATE/MIN.) | RESPIRATORY MIN. VOL. (ML./MIN.) | LEVORPHAN TARTRATE (MG.) | LEVALLORPHAN TARTRATE (MG.) | EFFECT UPON RESPIRATION (RATE/MIN.) | EFFECT UPON RESPIRATION (MIN. VOL. (ML./MIN.) |
|---|---|---|---|---|---|---|
| 1 | 18 | 9,750 | 10 | 1.0 | 8 | 4,500 |
| 2 | 16 | 8,750 | 6 | 0.6 | 8 | 4,500 |
| 3 | 20 | 9,600 | 8 | 0.8 | 7 | 4,250 |
| 4 | 16 | 8,400 | 6.6 | 0.66 | 8 | 5,000 |

All four patients receiving simultaneous administration of the drugs exhibited definite respiratory depression (Table III). In all patients in whom respiratory depression occurred or persisted with the 10:1 ratio, additional levallorphan tartrate provided further antagonism of the depression.

## DISCUSSION

It appears that this antagonist, administered after respiratory depression has been produced by levorphan tartrate, will effectively antagonize this depression when the drugs are given in a ratio of 10 units of depressant to 1 unit of antagonist. The fact that respiratory depression occurred when the antagonist was given prophylactically or simultaneously with levorphan indicates that if any protection is afforded in this ratio, it is incomplete. It may be that the 10:1 ratio used in this study is a borderline ratio.

When the dose of analgetic was four times and two times as great as the dose of antagonist, no significant inhibition of the maximum analgetic threshold rise was observed if the agents were given simultaneously. Also when levorphan tartrate was administered thirty minutes prior to levallorphan in a one to one ratio, no statistically significant alteration in maximum threshold rise was noted. When given fifteen minutes prior to the analgetic, a ratio of 1 unit of antagonist to 2 units of analgetic produced significant lowering of the threshold rise.

## SUMMARY

The problems inherent in measuring analgesia and levels of depression resulting from the use of depressant drugs prohibit definite conclusions. The results obtained, however, suggest that there may exist a difference between the amount of levallorphan tartrate needed to antagonize levorphan-induced respiratory depression and the amount needed to inhibit analgesia produced by the same analgetic drug in man. Additional studies are in progress to explore this possibility more completely.

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# SENSITIVITY OF COCCIDIOIDES IMMITIS TO 2-HYDROXY-STILBAMIDINE AND THE FAILURE OF THE DRUG IN THE TREATMENT OF EXPERIMENTAL COCCIDIOIDOMYCOSIS

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THE success of stilbamidine in the treatment of clinical blastomycosis has been reported by several investigators.[1-4] Chemotherapy with stilbamidine has been reviewed by Schoenbach, Miller, and Long,[2] and Snapper, Schneid, McVay, and Lieben.[5] These latter authors reported that 2-hydroxystilbamidine, a stilbamidine derivative, is less toxic and inhibited the growth of *Blastomyces dermatiditis* and *Histoplasma capsulatum* in vitro in very small concentrations.

Snapper[6] has been conducting clinical trials with 2-hydroxystilbamidine in several patients with disseminating coccidioidomycosis. Thus far, clinical results have been inconclusive as have also been our serologic evaluations. On the basis of Snapper's findings we undertook a study of the inhibitory potential of this drug against *Coccidioides immitis* in vitro and of its in vivo effect on coccidioidomycosis in mice.

## MATERIALS AND METHODS

1. *In Vitro.*—A stock solution of 2-hydroxystilbamidine* was made by dissolving 20 mg. of the compound in 20 ml. of sterile liquid asparagin medium.[7] The solution was not sterilized since it was found to be free of contamination.

Tenfold dilutions of the 2-hydroxystilbamidine solution were made in the asparagin medium so that varying amounts of the drug were contained in 9 ml. of the medium in each test tube. To each test tube was added 0.1 ml. of a spore suspension of *Coccidioides immitis* containing approximately 5,000 spores per milliliter. The test was set up in duplicate and allowed to incubate at 37° C. for twenty-one days. Observations of growth were recorded during this time.

Similar tests were performed in asparagin medium to which was added 5 per cent arginine hydrochloride since this amino acid may interfere with the action of the drug.[6]

2. *In Vivo.*—White mice, Namru strain, weighing twenty to twenty-five grams, were inoculated intraperitoneally with 0.5 ml. of a spore suspension of *Coccidioides immitis* strain Silveira. The inoculum contained 150 spores as determined by plate count. The 2-hydroxystilbamidine was prepared by dissolving the crystalline preparation in 6 per cent gum acacia as recommended by Snapper[6] so that 0.5 mg. of the drug was contained in 0.5 ml. The suspension was administered subcutaneously. In preliminary trials, 2.0 mg. of the drug inoculated daily caused rapid weight loss and early deaths. The 0.5 mg. dosage was the maximum which could be tolerated over a period of time.

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This work was conducted under the sponsorship of the Commission on Acute Respiratory Diseases of the Armed Forces Epidemiological Board and was supported by the Office of the Surgeon General, Department of the Army.

Received for publication Jan. 22, 1954.

*Lot No. CL-CY5354-CM and DD-C46537DE generously supplied by the Wm. S. Merrell Company, Cincinnati, Ohio.

The first dose of 0.5 mg. was given immediately after the mice were infected and repeated daily for ten days. After ten days the drug was given every other day because the control animals were losing weight. Control animals received an equal volume of 6 per cent gum acacia subcutaneously.

All animals which died were autopsied; the lungs, liver, spleen, and mesentery were examined for evidence of infection.

## RESULTS

Complete inhibition of growth in vitro for the period of observation (twenty-one days) was obtained when the organism was added to media containing 100 $\mu$g per ml. of 2-hydroxystilbamidine. In the medium prepared without arginine there was temporary inhibition with lower concentrations, but with further incubation growth occurred in these tubes. These results are presented in Table I. Similar results were obtained in vitro using another strain of Coccidioides recovered from a patient being treated by Snapper with 2-hydroxystilbamidine.

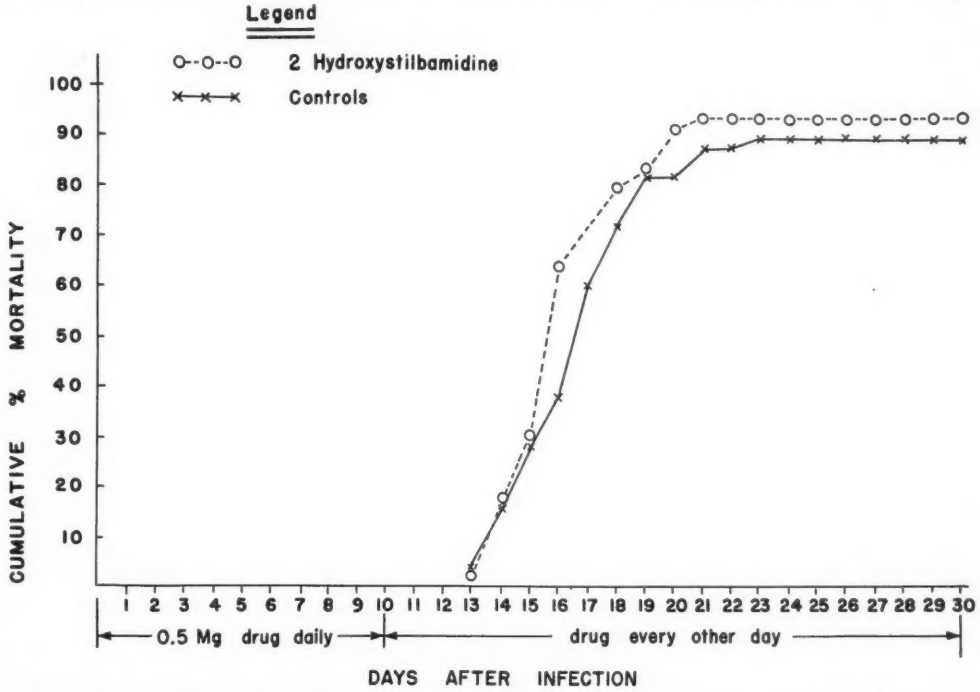


Fig. 1.—Cumulative percentage mortality in mice infected intraperitoneally with 150 Coccidioides spores of Silveria strain, fifty receiving 2-hydroxystilbamidine and fifty controls.

In Fig. 1 are shown the cumulative percentage of deaths for fifty mice which had received 2-hydroxystilbamidine and for an equal number of control animals. The drug did not reduce or delay the mortality in the treated group. A similar observation was made in a smaller group which had 2.0 mg. of the drug for a short period. The institution of drug therapy in 0.5 mg. dosage thirteen days prior to infection likewise failed to alter the course of deaths.

The lesions of treated and untreated mice were similar. All animals showed massive involvement of lungs, liver, spleen, and mesentery with coccidioidal nodules.

TABLE I. EFFECT OF 2-HYDROXYSTILBAMIDINE ON THE GROWTH OF COCCIDIOIDES IMMITIS IN ASPARAGIN MEDIUM
(Concentration of Drug Per Milliliter)

| DAY | 100 μG | | 10 μG | | 1.0 μG | | 0.1 μG | | 0.01 μG | | CONTROL NONE |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | *With arginine* | | | | | | |
| 3 | − | − | − | − | − | − | − | − | + | + | + |
| 4 | − | − | − | − | − | − | − | − | + | + | + |
| 7 | − | − | + | + | + | + | + | + | + | + | + |
| 8 | − | − | + | + | + | + | + | + | + | + | +++ |
| 10 | − | − | + | + | ++ | ++ | ++ | ++ | ++ | ++ | ++++ |
| 14 | − | − | + | + | +++ | +++ | +++ | +++ | +++ | +++ | ++++ |
| 16 | − | − | +++ | +++ | ++++ | ++++ | ++++ | ++++ | +++ | +++ | ++++ |
| 21 | − | − | +++ | +++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ |
| | | | | | *Without arginine* | | | | | | |
| 3 | − | − | − | − | − | − | + | + | + | + | + |
| 4 | − | − | − | − | + | + | ++ | ++ | ++ | ++ | ++ |
| 7 | − | − | − | − | + | + | ++ | ++ | ++ | ++ | ++ |
| 8 | − | − | − | − | + | + | ++ | ++ | ++ | ++ | ++ |
| 10 | − | − | − | − | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| 14 | − | − | − | − | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| 16 | − | − | + | + | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| 21 | − | − | + | + | +++ | +++ | ++++ | +++ | +++ | +++ | +++ |

## DISCUSSION

The administration of 2-hydroxystilbamidine in just below toxic levels to mice infected with *Coccidioides immitis* failed to demonstrate any beneficial effect. A similar failure of stilbamidine on experimental blastomycosis in mice has been reported.[8] Yet, in vitro, this drug has been shown to be inhibitory for *Blastomyces dermatiditis* and effective in the treatment of systemic and cutaneous blastomycosis in human beings. Our studies on the effects of 2-hydroxystilbamidine against *Coccidioides immitis* in vitro suggest that dosages required would be too large to be practical. While our in vivo failures are discouraging, there still remains a possibility of its clinical value for man. Analysis of treatment of patients with progressive disease will be necessary before a final decision can be made.

## SUMMARY

Inhibition of *Coccidioides immitis* by 2-hydroxystilbamidine, in vitro, was determined.

Mice were infected intraperitoneally with a spore suspension of *Coccidioides immitis* and treated with 2-hydroxystilbamidine. No beneficial effect of the drug was noted.

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# *LABORATORY METHODS*

## A STRIP-GRADIENT METHOD FOR IN VITRO ASSAY OF BACTERIAL SENSITIVITY TO ANTIBIOTICS PAIRED IN VARIOUS CONCENTRATION RATIOS

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CLINICALLY, there is need for a simple, rapid in vitro method for determining bacterial sensitivity to combinations of antibiotics. This is especially important where a microorganism is resistant to a single antibiotic, but may be sensitive to the same antibiotic combined with another drug or to other drug combinations. Such synergistic action of antibiotics in pairs is well known, and has been demonstrated both in vitro and in vivo.[1-5] Conversely, antibiotic antagonism also has been observed in vitro and in laboratory animals,[6-9] as well as clinically.[10]

At present, bacterial sensitivity to drug combinations is determined by tube dilution methods,[11, 12] in which assay end points are the lowest concentrations of the drugs giving complete inhibition of bacterial growth after relatively long incubation periods, or by more quantitative modifications of tube dilution tests, such as turbidometric assay[13] or actual plate counts of numbers of surviving organisms.[6, 13, 14, 15] Such methods, however, are time-consuming and complicated.

The strip-gradient technique here described was devised in an effort to provide a simpler, more rapid sensitivity test than the tube dilution methods now in use. The new procedure combines the Szybalski gradient plate technique[16-18] with the paper disc assay method,[19, 20] which has been modified by substituting an antibiotic impregnated paper strip for the disc. Strip-gradient plates may be used to indicate whether the action of drug pairs is synergistic or additive, antagonistic, or indifferent.

### MATERIALS AND METHODS

*Antibiotics.*—Stock solutions of crystalline penicillin G potassium* (1,000 units per milliliter), Terramycin hydrochloride* (1,000 μg. per milliliter), and streptomycin sulfate† (5,000 μg. per milliliter) were prepared with sterile distilled water, and diluted to the desired concentration at the time each test was performed. The stock solutions of penicillin and Terramycin maintained their potency for a period of at least one week when kept in the refrigerator; the stock solution of streptomycin sulfate still had the same potency after six months at 4° C.

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Received for publication, Dec. 15, 1952.

*Buffered penicillin and terramycin were supplied in diagnostic kits through the courtesy of Charles Pfizer & Co., Inc., Brooklyn, N. Y.

†Streptomycin sulfate, U.S.P.

In the strip-gradient technique, one antibiotic (primary) was incorporated in the gradient plate, and the other (secondary) was used to impregnate the paper strip. In the studies reported, streptomycin was used as the primary, and Terramycin or penicillin as the secondary antibiotic.

*Gradient Plates.*—The technique of Szybalski[16-18] was followed, using pour as well as streak plates. The appropriate sterile culture medium* for the organism under investigation was added to screw-capped tubes (16 by 150 ml.) in aliquots of 14.5 ml. and kept in a water bath at 48° C. As required, 0.5 ml. aliquots of aqueous dilutions of primary antibiotic were added to each tube of culture medium, and the contents mixed and poured into sterile 100 ml. pressed bottom petri dishes.

Gradient plates were prepared by pouring two 15 ml. layers of agar. The first layer of agar containing no antibiotic was permitted to harden with the dish slanted just sufficiently to cover the entire bottom of the plate. The second agar layer, containing the desired upper assay limit concentration of the primary antibiotic, was poured after placing the dish in the normal horizontal position. The range and slope of the antibiotic gradient could be further adjusted by also incorporating another concentration of the primary drug in the bottom agar layer, thereby changing the ratio of drug concentrations in the layers.

*Bacterial Inoculum.*—A suspension of the test organism was heavily streaked over the complete surface of the hardened gradient plate, using either a wire spreader or a cotton swab moistened with the culture. An alternate procedure that provided a more uniformly distributed inoculum was the seeding of 0.02 ml. of bacterial culture into each of the two agar layers immediately before they were poured. The bacterial inocula used were twenty-four-hour cultures of: *Micrococcus pyogenes var. aureus* (Smith) and an in vitro derived streptomycin resistant variant[11] in nutrient broth (Difco), and *Streptococcus viridans* and *Streptococcus pyogenes* (Group A, type 12) in heart infusion broth (Difco) enriched with 4 per cent defibrinated sheep's blood. The strain of *Strep. viridans* was isolated from the throat of a debilitated adult woman with "chronic sore throat."† The strain of *Strep. pyogenes,* Group A, type 12 was isolated from the throat of a presumably healthy child in the course of a streptococcal survey of Miami school children.

*Paper Strip.*—A sterile, dry strip (0.4 by 8.5 mm.) of filter paper (Whatman No. 42) was immersed in a tube containing an aqueous dilution of the secondary antibiotic prepared in the concentration desired as the upper assay limit. The strip was then lifted from the solution with a sterile, pointed forceps. Excess droplets of antibiotic were removed by thoroughly draining the strip against the upper portion of the tube, or by placing the strip on a sterile glass slide and lightly applying the edge of another sterile glass slide along the strip surface. The paper strip impregnated with the secondary antibiotic was then centrally placed across the gradient agar plate so that it fell along the axis of gradual proportionate change of concentration of the primary drug. Plates were incubated at 37° C. for eighteen to twenty-four hours.

*Reading of Strip-Gradient Plates.*—Reading of the strip-gradient plates is based upon the fact that the primary antibiotic in the gradient plate diffuses vertically so that the drug is diluted in proportion to the thickness ratio of the agar layers, and a uniform concentration slope is established. The secondary antibiotic diffusing from the paper strip sets up a steep logarithmic concentration gradient throughout the areas adjacent to the strip. The strip-gradient plate thus graphically depicts the effects upon a particular bacterial population of two antibiotics in the various combination ratios resulting within the limits of the concentrations selected for the test.

In general, there are two areas of the plates which must be examined, as shown in Fig. 1. Area A contains only the primary antibiotic. It gives a quantitative in vitro assay of the sensitivity of a bacterial culture to the drug and also affords visual distinction of the inhibition pattern. This area yields essentially the results obtainable with the Szybalski gradient plate.

*Blood agar base (Difco) was used for the studies on *Micrococcus pyogenes var. aureus* (Smith); 4 per cent defibrinated sheep's blood was added to the base medium for the growth of *Streptococcus viridans* and *Streptococcus pyogenes.*

†Obtained through the courtesy of La Huis Laboratories, Miami, Fla.

Area B contains both antibiotics in all the possible combination ratios resulting from the diffusion of each drug within the limits set up by the initial concentrations used. The limits of area B are $B_L$ and $B_H$, both of which have the same concentration of secondary antibiotic, but a concentration of primary antibiotic rising from a minimum at $B_L$ to a maximum at $B_H$.

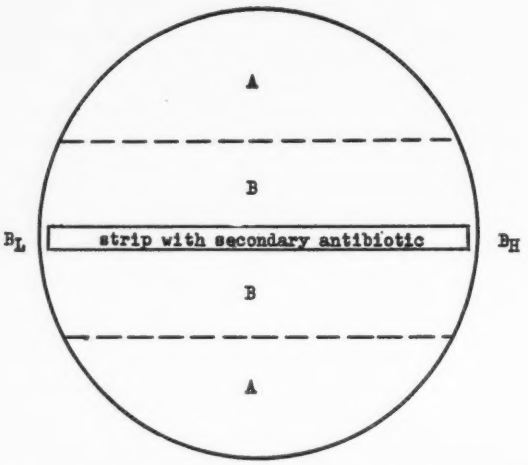



Fig. 1.—Guide for reading results of strip-gradient plate.

The areas of growth or zones of inhibition about the paper strip indicate the relative degree of bacterial sensitivity to the antibiotics in the particular concentration ratios at each point. Thus a more precisely defined picture of the synergistic or additive, the antagonistic, or the indifferent action of two combined antibiotics is obtained.

*Dilution and Plating Controls.*—The strip-gradient plate tests with *M. pyogenes var. aureus* (Smith) and *Strep. viridans* were repeated using a variation of dilution and plating techniques.[6, 13] To a series of tubes containing 8.9 ml. of culture medium* were added 0.1 ml. amounts of aqueous solutions of each of two antibiotics in the concentration ratios desired, and 0.1 ml. of a twenty-hour culture of the test organism grown in the same medium, with the addition of sterile distilled water to a final volume of 10 ml. The number of viable organisms in these assay tubes was determined immediately, and after incubation at 37° C. for 2, 5, 8 and 24 hours in the case of the staphylococcus, and for 2, 6, and 24 hours in the case of the streptococcus, by the preparation of pour plates of blood agar base (Difco†) from 0.1 ml. aliquots appropriately diluted in sterile distilled water. The plates were incubated at 37° C. for 24 hours and colony counts made.

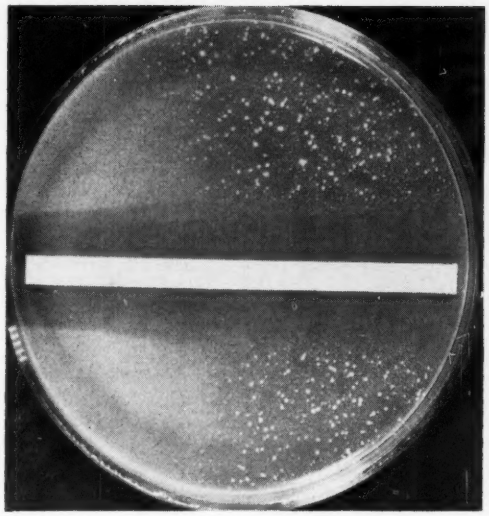
## RESULTS

Figs. 2 to 6 illustrate the results obtained with the strip-gradient plate. It can be seen that the technique is suitable for demonstrating the antagonistic (Fig. 3) and the synergistic or additive effects (Figs. 2, 4, and 5) of paired antibiotics, as well as the antibacterial action of individual drugs.

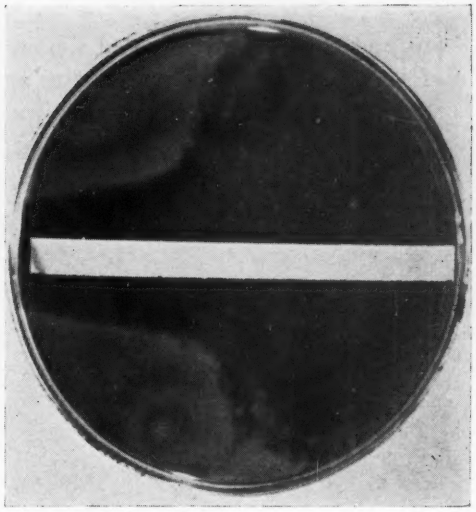
The pattern of bacterial growth observed in the strip-gradient plate varied depending on which of an antagonistic pair of antibiotics was placed on the strip and which was incorporated in the agar. In our studies with *M. pyogenes var. aureus* (Smith), Terramycin appeared to negate almost entirely the growth-inhibiting action of streptomycin when the position of the

*Nutrient broth (Difco) for the staphylococcus, and heart infusion broth (Difco) containing 4 per cent defibrinated sheep's blood for the streptococcus.

†Plus 4 per cent defibrinated sheep's blood for the streptococcus.



*A.*

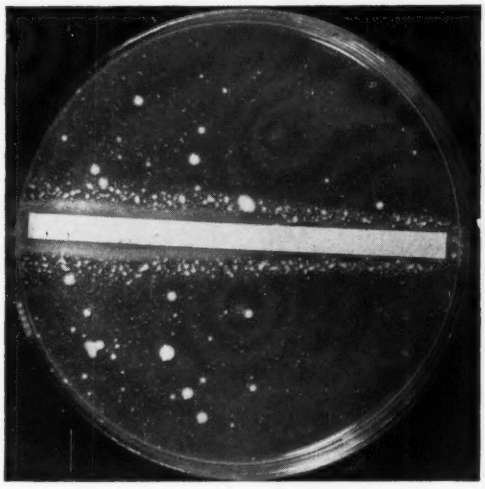


*B.*

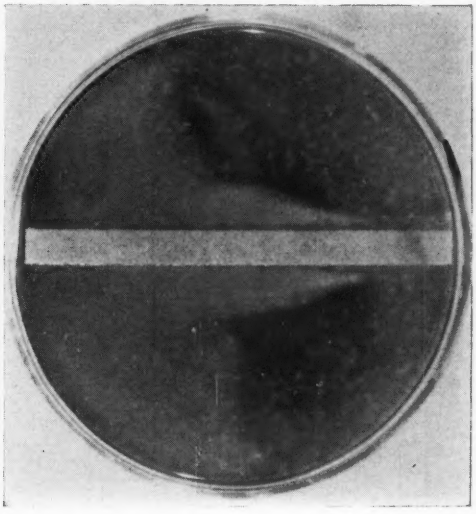
Fig. 2.—Synergistic or additive effect of penicillin-streptomycin combinations. Note: (1) flaring zone of inhibition of growth in area B despite the uniform distribution of penicillin along the strip; and (2) gradual tapering off of growth in area A coinciding with increasing concentrations of streptomycin.

*A. M. pyogenes var. aureus* (Smith) in a strip-gradient pour plate of blood agar base (Difco). Concentrations of antibiotics: penicillin on paper strip, 10 units per milliliter; streptomycin in gradient plate, within limits of 0 to 3 μg per milliliter.

*B. Strep. pyogenes* (Group A, type 12) in a strip-gradient pour plate of blood agar base (Difco) enriched with 4 per cent defibrinated sheep's blood. Concentrations of antibiotics: penicillin on paper strip, 40 units per milliliter; streptomycin in gradient plate, within limits of 0 to 20 μg per milliliter.



*A.*



*B.*

Fig. 3.—Antagonistic action of Terramycin-streptomycin combinations: Note: (1) in area A, gradual diminution of bacterial growth (and areas of hemolysis in 3*A*), with increasing concentrations of streptomycin; (2) the bacterial growth along the paper strip despite the presence of streptomycin in bacteriostatic concentrations; (3) the gradual decrease of bacterial growth along the strip from $B_L$ to $B_H$, as the concentration of streptomycin increases; and (4) in 3B, at $B_H$ the almost complete absence of effect of Terramycin on *Strep. pyogenes* in the presence of relatively high concentrations of streptomycin.

*A. M. pyogenes var. aureus* (Smith) in a strip-gradient pour plate of blood agar base (Difco). Concentrations of antibiotics: Terramycin on paper strip, 10 μg per milliliter; streptomycin in gradient plate, within limits of 0 to 5 μg per milliliter.

*B. Strep. pyogenes* (Group A, type 12) in a strip-gradient pour plate of blood agar base (Difco) enriched with 4 per cent defibrinated sheep's blood. Concentrations of antibiotics: Terramycin on paper strip, 60 μg per milliliter; streptomycin in gradient plate, within limits of 0 to 20 μg per milliliter.

antibiotics was reversed and the streptomycin was placed in the strip instead of in the plate. The interfering agent would thus appear to be Terramycin rather than streptomycin. This was also apparent when the same antibiotics were tested by the tube dilution and plating technique (Fig. 8).

Fig. 4.

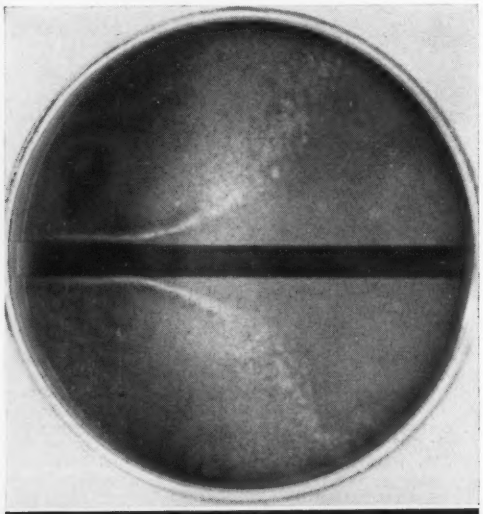


Fig. 5.

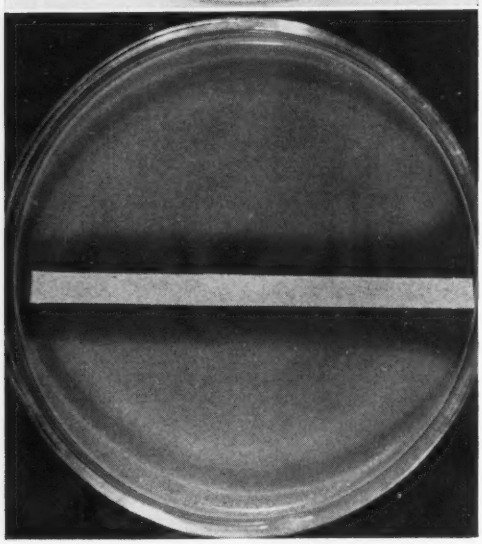
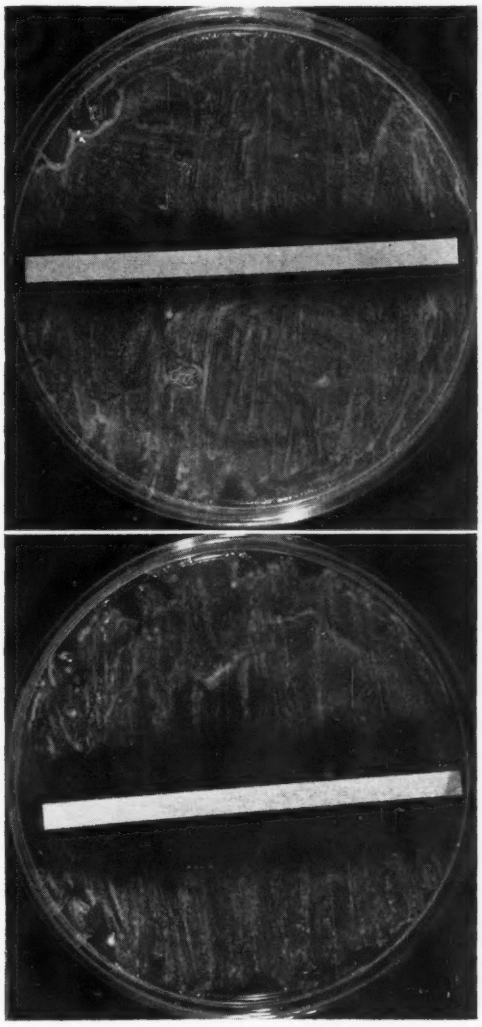


Fig. 4.—Synergistic or additive action of Terramycin-streptomycin combinations on *Strep. viridans* in a strip-gradient pour plate of blood agar base (Difco) enriched with 4 per cent defibrinated sheep's blood. Concentrations of antibiotics: Terramycin on paper strip, 10 μg per milliliter; streptomycin in gradient plate, within limits of 0 to 14 μg per milliliter. Note: (1) the flaring zone of inhibition of growth in area B, as the concentration of streptomycin increases and the concentration of Terramycin about the strip remains constant; (2) in area A, the limited extent to which the test organism is sensitive to streptomycin only; and (3) the low inhibitory action of Terramycin in the presence of small concentrations of streptomycin at $B_L$.

Fig. 5.—Action of penicillin (10 units per milliliter) in paper strip on an in vitro derived streptomycin-resistant strain of *M. pyogenes var. aureus* (Smith) in a gradient pour plate of blood agar base (Difco) containing streptomycin within limits of 0 to 5 μg per milliliter. Note: (1) the relative ineffectiveness of streptomycin alone in inhibiting bacterial growth in area A; (2) the inhibiting action of penicillin evident in area B; and (3) the slight widening of the area of inhibition of growth, indicative of a somewhat additive or synergistic effect of the two drugs at the concentration ratio achieved at $B_H$.

For the test strains of *M. pyogenes var. aureus* (Smith) and *Strep. viridans,* patterns of antibiotic action observed by the strip-gradient method with penicillin-streptomycin and Terramycin-streptomycin combinations were found to correlate with those of the dilution-plating technique (Figs. 7, 8, and 9). The optimal correlation between the two methods was evident when the numbers of organisms viable after exposure to drug combinations for approximately five to eight hours were plotted.

*A.*



*B.*

Fig. 6.—Streaked strip-gradient plates of blood agar base (Difco), showing the actions of (a) Terramycin (10 μg per milliliter) and (b) penicillin (10 units per milliliter) in the paper strip, and streptomycin (within the limits of 0 to 10 μg per milliliter) in the gradient plates, on an in vitro derived streptomycin-resistant strain of *M. pyogenes var. aureus* (Smith). Note: (1) the relative zones of inhibition of bacterial growth in area B in both plates; and (2) in plate (b), the slight widening of the growth-inhibition area at $B_H$ and slight narrowing at $B_L$.

It is recognized that the decreasing numbers of surviving organisms acted upon by a secondary antibiotic along the axis $B_L$ to $B_H$ should be considered in evaluation of a synergistic or additive growth-inhibition flare. This would appear to be of little significance, however, in view of the correlation of the strip-gradient plates with the tube dilution and plating technique.

Synergism was not distinguished from additive effect in the illustrations presented. Such differentiation may be made by fulfilling the criteria for synergism of Jawetz and Gunnison,[21] that is, "only one [drug] need exhibit measurable biologic effect on the concentration entering into synergism. The other must show some effect but only in a much higher concentration."

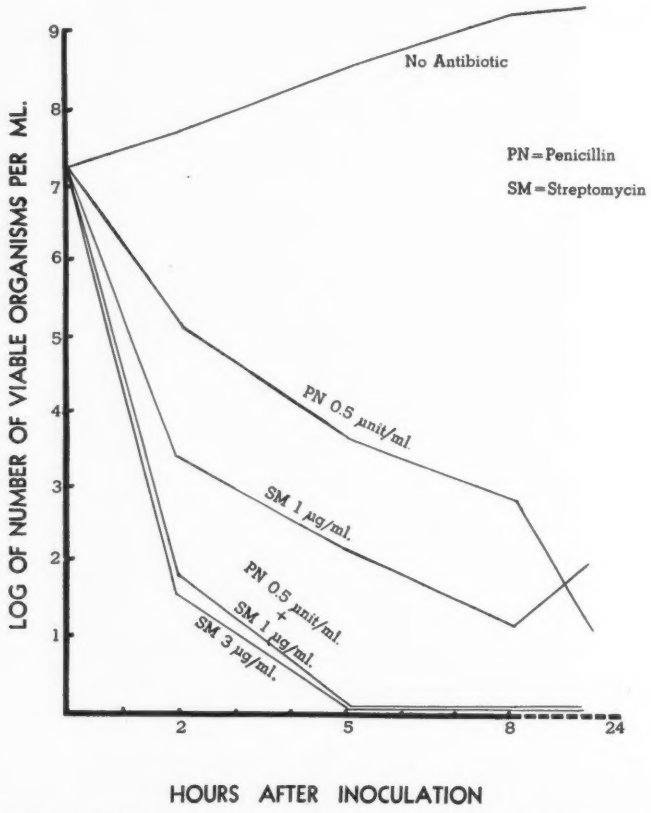



Fig. 7.—Synergistic or additive effect of penicillin-streptomycin combinations on *M. pyogenes var. aureus* (Smith), as demonstrated by tube dilution and plating.

## DISCUSSION

Simultaneous editorials in the *New England Journal of Medicine*[22] and the *Journal of the American Medical Association*[23] have re-emphasized the many factors that may influence the results of in vitro tests for bacterial susceptibility to antibiotics. These factors include, among others, the following: (a) type and composition of the culture medium; (b) pH of the culture medium; (c) size and age of the bacterial inoculum; (d) antibiotic-inactivating

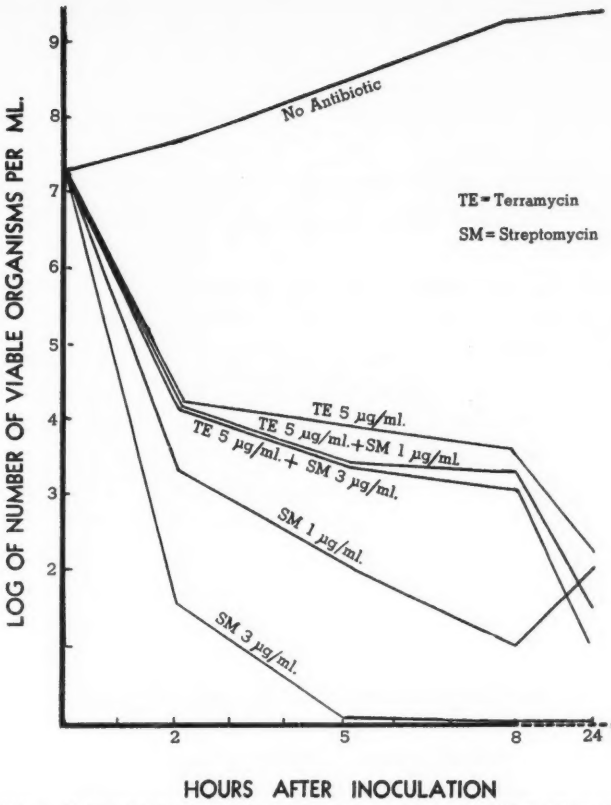



Fig. 8.—Pattern of survival of *M. pyogenes var. aureus* (Smith) after exposure to Terramycin-streptomycin combinations. Note the interference of Terramycin with the bactericidal effect of streptomycin during the first eight hours of incubation and the diminution of interference on twenty-four hours of incubation.

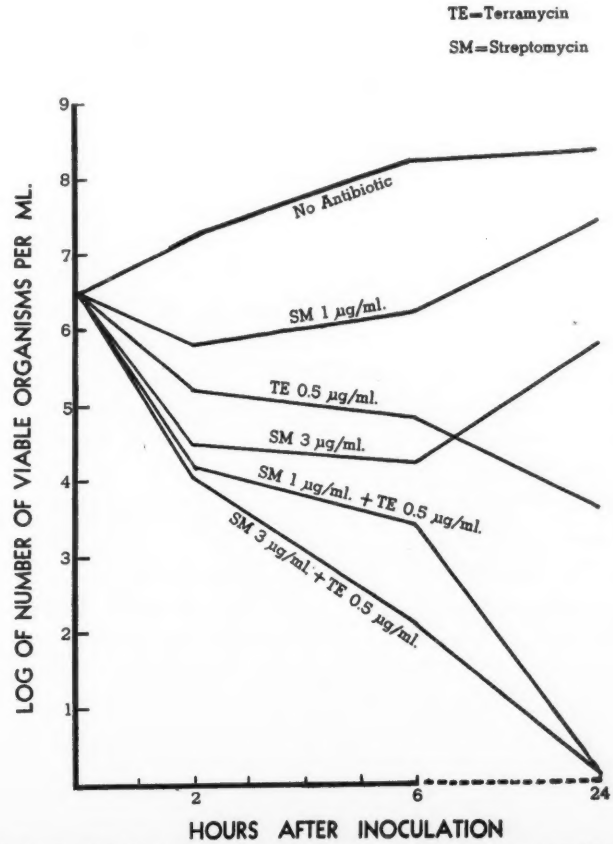



Fig. 9.—Synergistic or additive effect of Terramycin-streptomycin combinations on *Strep. viridans*, as demonstrated by tube dilution and plating.

factors present in the culture medium either initially or as a result of bacterial growth; (e) incubation period; (f) concentration and stability of the antibiotic.

Any of these factors, alone or in combination, may affect the outcome of any in vitro sensitivity test, depending on the organism and the antibiotic tested. In the assay of bacterial sensitivity by the paper disc method, as ordinarily performed, the results have been shown to be modified markedly also by the rate of diffusion of the antibiotic through the agar and the absolute quantity of the antibiotic absorbed and retained by the paper disc.[19, 24]

The interpretation of the strip-gradient method is subject to all of the above factors. It compares favorably, however, with the tube dilution and plating techniques now in use. It yields qualitatively duplicable results showing the relative in vitro sensitivity of a given organism to a range of concentrations of particular drug pairs. It permits the study in one plate of patterns of early bacteriostatic and bactericidal action of antibiotics singly or in pairs, which are often missed entirely by tube dilution techniques and are demonstrable only by platings of a series of tubes after various intervals of incubation. More precise assay end points of any particular level of concentration ratios in a strip-gradient plate may be produced by setting up other strip-gradient plates containing the specific concentrations of antibiotics at such levels. Thus the antibiotic action observed as part of the over-all pattern can be duplicated in greater detail.

It has been observed that, dependent upon the absolute concentrations in which they are used and their ratio to each other, two antibiotics may be either antagonistic, indifferent, additive, or synergistic in action against the same bacterial culture.[15] In addition, certain drug pairs may be synergistic or additive in action on one microorganism, antagonistic on another, and indifferent on a third.[21, 25-27] Thus, we found Terramycin and streptomycin to be antagonistic against *M. pyogenes var. aureus* (Smith) which was highly sensitive to both drugs, and synergistic or additive against a moderately streptomycin-sensitive test strain of *Strep. viridans*. Individual strains within a given bacterial species or variants derived from one strain may show decided differences in susceptibility to a particular drug pair.[21, 25, 26] The strip-gradient procedure, by indicating the type of action resulting at each concentration ratio of two antibiotics within the dilution ranges set for the test, affords a graphic picture of the changes in bacterial response to a pair of drugs at different concentration ratios.

The clinical use of laboratory tests for the determination of bacterial sensitivity to various antibiotics or combinations of antibiotics has been based on the assumption that the effectiveness of the drugs in vivo would parallel that observed in vitro. This does not take into account, however, the factors both in the host and in the invading bacterium which may alter in vivo, the effectiveness an antibiotic displays in vitro. Variations of antibiotics in their absorption, diffusion, and excretion at different sites in the host, as well as the number, concentration, and metabolic state of the infecting microorganism

must be considered in any evaluation of a potential therapeutic agent.[25, 28, 29, 30] The length of time antibiotics are administered and maintained at therapeutic levels is also a determining factor in the type of reaction observed in vivo.[8, 28, 31] How the in vitro findings of the strip-gradient plate correlate with the clinical effects of antibiotic combinations remains to be determined.

Within its limitations of interpretation, it is believed that the strip-gradient method can be used for the same purposes proposed for other in vitro tests: (a) As a general qualitative guide to distinguish susceptible from nonsusceptible organisms[29]; (b) As an index of the relative sensitivity of different organisms or of different strains of the same species to any given antibiotic[29]; (c) As a guide in the early therapeutic approach[30]; (d) As a safeguard during treatment.[30]

The Szybalski gradient plate component makes it possible also to determine: (a) Single plate serial assay of bacterial sensitivity to a single antibiotic.[16-18] (b) The pattern of resistance displayed by a particular microorganism in the presence of varying concentrations of a single antibiotic.[16-18]

Incorporation of an antibiotic-impregnated paper strip in the gradient plate provides, in addition: (a) A simple, rapid technique for testing bacterial sensitivity to a pair of antibiotics. (b) A graphic indication as to the synergistic or additive, indifferent, or antagonistic action of two antibiotics at various concentration ratios.

SUMMARY

A strip-gradient plate technique for the in vitro assay of bacterial sensitivity to antibiotics singly or in pairs has been described and compared with a tube dilution-plating method. The new procedure combines the Szybalski gradient plate with an antibiotic-impregnated paper strip. The combination of these components into one test makes possible the determination in one plate of synergistic, additive, indifferent, or antagonistic action of paired antibiotics at various concentration ratios. Studies on test strains of *M. pyogenes var. aureus* (Smith) and *Strep. viridans* indicate that the patterns observed with strip-gradient plates correlate qualitatively with those obtained with more time-consuming, complicated tube dilution-plating methods. The strip-gradient plate technique is simple, rapid, and reproducible. Its clinical value, limitations, and interpretation are discussed.

We wish to express our appreciation for the technical assistance of Miss Alba Colon.

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# COLORIMETRIC DETERMINATION OF PROTEIN-BOUND, WATER-INSOLUBLE SULFHYDRYL GROUPS WITH THE BARRNETT-SELIGMAN REAGENT

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THIS paper deals with the adaptation of Barrnett-Seligman's histochemical method for the direct quantitative estimation of protein-bound, water-insoluble sulfhydryl groups. In a previous work we described the adaptation of Bennett's histochemical reagent for the quantitative estimation of such groups.[1] The present method makes use of the histochemical reagent, 2,2′-dihydroxy-6,6′-dinaphthyl disulfide which, in the presence of sulfhydryl groups, becomes reduced by splitting of the disulfide bond. One of the split products combines with the sulfhydryl groups of the proteins and is visualized by coupling with tetrazotized diorthoanisidine.[2, 3]

Originally, we attempted to determine the amounts of protein-bound dye after incubation of the sulfhydryl-containing proteins with the reagents. In spite of numerous attempts, we did not succeed in completely separating the dye from the protein-dye complex and in bringing all of it into solution. It was found more accurate and convenient to estimate the amounts of the free dye, by measuring the reduction in the color of the supernatant solution after incubation with sulfhydryl-containing proteins. This color was then compared with the color obtained after incubation of the reagents with a standard denatured egg-white powder, the sulfhydryl content of which had been determined previously by another method.

## MATERIALS AND METHODS

1. *Reagents.*—(a) 2,2′-dihydroxy, 6,6′-dinaphthyl disulfide† (henceforth called Reagent I). One milligram of the reagent is freshly dissolved in 4 ml. absolute alcohol and diluted to 10 ml. with 0.1 M barbiturate buffer, pH 8.5; (b) 10 per cent aqueous Duponol C (sodium lauryl sulfate); (c) tetrazotized diorthoanisidine (Reagent II). One milligram of the reagent freshly dissolved in 2 ml. water; (d) standard denatured egg-white powder. Powdered egg white (Merck) is dissolved in water and precipitated with absolute alcohol. The precipitate is repeatedly washed with absolute alcohol and water. After drying, the protein is pulverized in a mortar and passed through a 200 mesh sieve.

2. *Procedure.*—The dried proteins to be analyzed are passed through a 200 mesh sieve. Samples of 5, 10, and 15 mg. each of the standard egg-white powder and of the protein to be analyzed are weighed in test tubes. To each tube 2 ml. of Reagent I is added. The test tubes are incubated for one hour in a water bath at 50° C., with frequent shaking.

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From the Department of Dermatology (Donald M. Pillsbury, Director), University of Pennsylvania School of Medicine.
Aided by a grant from the National Institutes of Health, U. S. Public Health Service, No. G-3601.
Received for publication, Dec. 17, 1953.
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At the end of this period, the tubes are centrifuged and 0.2 ml. of the supernatant is measured into test tubes containing 0.5 ml. of Duponol solution. To this mixture 0.5 ml. of Reagent II is added and the contents of the tubes are mixed thoroughly. An immediate purple color develops which is stable for several hours. The contents of the test tubes are diluted with 10 ml. water and the color is measured in a spectrophotometer at a wavelength of 560 mμ, against a water blank. From the readings the values corresponding to 1 mg. of protein are calculated. These values are compared with the figure corresponding to that of 1 mg. standard egg white. (Fig. 1).

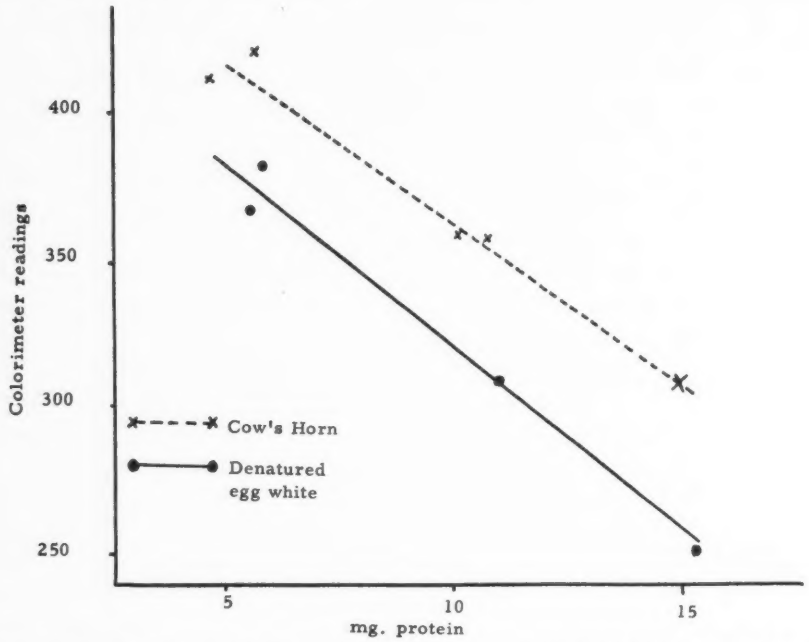


Fig. 1.—Uptake of Barrnett-Seligman reagent by denatured egg-white powder and cow's horn.

## RESULTS

With this method, the presence of considerable amounts of sulfhydryl in keratins[4] was confirmed. The amounts of sulfhydryl in mM $\times 10^{-5}$ per 100 mg. dry weight of protein, as determined with Bennett's reagent after one hour's incubation with Duponol at 40° C. were as follows: denatured egg white: 456, cow's horn: 431, psoriasis scales: 220. (Ratio: 23:21.5:11.) With the present method the corresponding ratios were: 24:22:12.

When tested with the present method, urease yielded 28 per cent of the sulfhydryl content of egg white; the corresponding figure with Bennett's reagent was 13 per cent.

The size of the particles in the samples to be analyzed influenced the readings only when the particles were coarse. When particles of relatively small size were used for determination, further diminution of the size did not give rise to higher values. This limiting size was well above the size obtained by passing the proteins through a 200 mesh sieve. Thus, non-pulverized flaking horny epidermal scales from a patient with psoriasis gave the same readings regardless of whether they were passed through a sieve or not.

## DISCUSSION

Because of its solubility characteristics, the Barrnett-Seligman reagent can be used only for the determination of water-insoluble sulfhydryl groups. This feature compares unfavorably with Bennett's reagent which forms a precipitate with both water-soluble and water-insoluble natural sulfhydryl compounds. The advantage of the present method over Bennett's modified technique is the commercial availability of the reagents used. The disadvantages are the more limited applicability, lesser sensitivity, and the need for larger amounts of test material.

In the keratins tested, the Barrnett-Seligman reagent combined with immediately, as well as potentially available sulfhydryl groups. This assumption is supported by the following findings. (a) Masked sulfhydryl groups in keratins, when made available by treatment with Duponol, react with Bennett's reagent.[5] The ratio of the values determined in this way corresponds with those estimated with the present method. (b) When, after reacting with the Barrnett-Seligman reagent, powdered proteins were subjected to Bennett's reagent in the presence of Duponol, no reaction occurred between the proteins and Bennett's reagent. This lack of reactivity was interpreted as indicating that all the available sulfhydryl groups had already combined with the Barrnett-Seligman reagent.

With the *p*-chloromercuribenzoic acid titration method, Hellerman and associates[6] recovered more than twice as much sulfhydryl in urease as in denatured egg white. In our findings, both Bennett's and Barrnett's and Seligman's reagents combined with fewer sulfhydryl groups of this enzyme.

## SUMMARY

The adaptation of Barrnett-Seligman's histochemical method for the quantitative colorimetric estimation of protein-bound, water-insoluble sulfhydryl groups is described. This new technique was compared with a previously described method, based on Bennett's histochemical sulfhydryl reagent. The advantages of the new method are its simplicity and the commercial availability of the reagents employed.

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# SIMPLIFIED PROCEDURE FOR DETERMINATION OF PBI$^{131}$ IN BLOOD

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THIS is one of a number of routine diagnostic studies, using radioactive isotopes, which have recently been revised in this laboratory.[1] Increased acceptance of these tests has caused us to initiate procedures which can be run on large numbers of patients, routinely, serially, and with negligible radiologic exposure. As indicated earlier, scintillation counters, new handling techniques, and disposable materials have facilitated our aims.

Herein a technique is described, as a part of thyroid status evaluation, for preparing protein-bound-iodine$^{131}$ samples for a scintillation well counter. The rationale and interpretation of the results of this test have been discussed elsewhere.[2] As many as twenty-four samples have been prepared and counted by a single technician in one four-hour period; many more than previously accomplished with dried samples counted under an end mica window geiger tube.

The tracer dose employed is 50 $\mu$c of NaI$^{131}$, administered orally. Using this dose, statistically accurate counting (P.E. of ± 2 per cent) was obtained for fifteen-minute counting periods.

## METHOD

**Material:**

50$\mu$c NaI$^{131}$*
10 per cent Trichloracetic Acid
40 per cent Trichloracetic Acid

**Apparatus:**

Scintillation well counter, NaI(Th) using modified Morton[3] circuit for 300-volt plateau.
Plastic test tubes,† ½ inch O.D. by 4 inch length with metallic screw cap.
Stirring rod, stainless steel, ¼ inch O.D. by 6½ inch length.

**Procedure:**

a. Pipet 5 ml. plasma into plastic test tube.
b. Add 1.5 ml. 40 per cent trichloracetic acid and stir thoroughly.
c. Centrifuge at 500 g for 10 minutes.
d. Add 5 ml. 10 per cent trichloracetic acid, stir thoroughly, centrifuge, and decant supernatant.
e. Repeat the above step two more times, making a total of three washes. In the final centrifugation, spin for 10 minutes at maximum speed to pack protein as densely as possible.

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Received for publication Feb. 9, 1954.
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f. Count final precipitate in scintillation well counter for 15-minute period.

g. From the total efficiency of the counter (35 per cent for $I^{131}$) a conversion factor of 1.93 by $10^5$ is derived.* The product of the latter and the net counts per fifteen minutes gives the $PBI^{131}$ in microcuries per liter of plasma corrected to time of administration (24 hours decay).

The plastic tubes are lowered into the crystal phosphor by means of a bar magnet holding the metal cap. The tubes are discarded after use and offer no problem in radiologic exposure. It has been found that the three washes remove at least 95 per cent of the non-protein-bound iodine.

It can be seen that with this technique, conversion ratios[4] can be simply determined with very little extra effort. Herein, 1 ml. of whole plasma is counted in the well counter. The ratio of the microcuries of $PBI^{131}$ per liter of plasma to the net microcuries of inorganic $I^{131}$ per liter of plasma gives the conversion ratio.

## SUMMARY AND RESULTS

In Table I are noted the results obtained from seventy-five consecutive patients upon whom the previously mentioned technique was used.

TABLE I

| CLASS | MEAN | S.D. | RANGE |
|---|---|---|---|
| Hyperthyroid | 0.135 $\mu$c/L | ±0.05 | 0.08 to 0.23 $\mu$c/L |
| Euthyroid | 0.028 $\mu$c/L | ±0.016 | 0.007 to 0.064 $\mu$c/L |
| Hypothyroid | 0.029 $\mu$c/L | ±0.015 | 0.008 to 0.064 $\mu$c/L |

From Table I, a sharp and clear demarcation of hyperthyroid and euthyroid states can be delineated. No such demarcation can be seen between euthyroid and hypothyroid conditions; such inability to discriminate between the two states with protein-bound radioiodine studies has generally been observed by other workers. This test performed in conjunction with the external thyroid pick-up of $I^{131}$ has been very useful in the diagnostic evaluation of patients with thyroid disease.

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*The conversion factor is obtained from the following:

$$\frac{1}{d/\mu c/15'} \times \text{eff.} \times \text{decay ftr.} \times 200 \text{ ml.}$$

# CONCERNING THE IDENTIFICATION OF CLOSTRIDIUM PERFRINGENS LECITHINASE IN BODY FLUID

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ATTEMPTS have been made to identify and assay the alpha toxin of *Clostridium perfringens* in body fluids as an indication of the presence of that microorganism, as well as to establish its possible role in the pathogenesis of certain types of disease.[1-7] Commonly, the technique of identification and assay of the alpha toxin involves measuring its lecithinase activity and showing that perfringens antitoxin inhibits it. Macfarlane and associates,[8] Macfarlane and Knight,[9] and Van Heyningen[10] have studied the reaction of this lecithinase with a solution of egg yolk as a substrate and have utilized this reaction for titration purposes. Perfringens lecithinase splits the lecithovitellin of yolk to produce a turbidity which, upon standing or centrifugation, rises to the surface of the liquid.[8, 9] The products of the reaction include soluble phosphorylcholine and an insoluble diglyceride. This lecithinase requires calcium ions (or certain other bivalent ion substitutes) for suitable action on the substrate.[8-11]

Attempts were made in this laboratory to measure lecithinase activity attributable to *Cl. perfringens* in body fluids of dogs having undergone experimental abdominal surgical procedures. In some of the samples of body fluid examined, at first, there appeared to be definite lecithinase activity. By applying a number of constraints involving known properties of lecithinase on the test systems, however, it was discovered that lecithinase was not being measured. It appeared that body fluids, especially from diseased tissues, at times possess substances which superficially mimic the activity of perfringens alpha toxin. This report deals with the methods found suitable for eliminating false positive tests for lecithinase activity in body fluids.

## MATERIALS AND METHODS

For comparative purposes, two samples of perfringens alpha toxin were employed; one was a purified product generously supplied by the Eli Lilly & Company, and the other was prepared in our laboratory. They reacted in an identical manner.

The body fluids examined for lecithinase activity consisted of samples of peritoneal exudate and bowel contents collected at various intervals from dogs experiencing experimental intestinal strangulation. Six of the twelve dogs studied have been fully described by Cohn and associates.[7]

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Received for publication, Feb. 23, 1954.

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The technique of Van Heyningen[10] was used to assay alpha toxin activity and to prepare the egg-yolk solution. Turbidities were measured with a Klett-Summerson photoelectric colorimeter. In early experiments, the test mixtures were incubated for 2 hours at 37° C. before making turbidity measurements. Because of the low level of activity found, in all of the remaining experiments, the incubation period was increased to 24 hours in order to detect much smaller amounts of lecithinase in the body fluids. Roccal* was added to the test mixture to a final concentration of 1:375,000 of active substance in order to inhibit growth of most bacteria that might be present. Many of the turbid mixtures were examined for the presence of bacterial cells by stained smears. Detectable bacterial cells were not observed in any of the incubated mixtures.

With this test, the samples of known perfringens alpha toxin gave good turbidity in 2 hours and heavy turbidity in 24 hours.

## RESULTS

It was found that Roccal in the concentration used had no adverse effect on the reaction system when known preparations of lecithinase and lecithovitellin solution were mixed. Table I presents data to show that Roccal does not inhibit lecithinase activity.

TABLE I. EFFECTS OF ROCCAL AND SODIUM FLUORIDE ON LECITHINASE ACTIVITY IN THE PRESENCE OF LECITHOVITELLIN SOLUTION

| TUBE | ROCCAL (ML.) | Na F (ML.) | LECITHINASE (ML.) | TURBIDITY (KLETT UNITS) |
|---|---|---|---|---|
| 1 | 0.1 | 0.1 | | 50 |
| 2 | 0.1 | | | 31 |
| 3 | 0.1 | 0.1 | 0.5 | 51 |
| 4 | 0.1 | | 0.5 | 475 |
| 5 | | 0.1 | | 47 |
| 6 | | | | 28 |
| 7 | | 0.1 | 0.5 | 31 |
| 8 | | | 0.5 | 475 |

All tubes brought to constant volume by the addition of the requisite amount of saline solution.

Upon finding that dog fluids often did produce marked turbidity in twenty-four hours of contact with the substrate solution, it was believed desirable to apply two tests of the properties of lecithinase to the systems in order to have greater assurance that the turbidity was actually caused by lecithinase. Accordingly, since it was known that calcium ions are needed for lecithinase activity, all tests were done in duplicate but with 0.1 ml. of 0.07 M sodium fluoride added to one, and a corresponding volume of saline solution added to the other. Table I also demonstrated that this concentration of sodium fluoride inhibits known lecithinase activity completely. It was a constant finding that tubes containing sodium fluoride and yolk solution gave a slightly greater turbidity than corresponding tubes containing saline solution substituted for the fluoride (compare tubes 1 and 2 in Table I). This effect is probably caused by the small amount of calcium fluoride precipitated.

A second test of lecithinase activity involved the ability of lecithinase to split fat from lecithovitellin. This was demonstrated by centrifuging the tubes

*Roccal—Winthrop-Stearns brand of 10 per cent alkyl-dimethylbenzyl ammonium chloride.

after making the turbidity readings. If some or all of the floccules present rose to the surface, the presence of free fat was indicated, while if the floccules sedimented to the bottom of the tube, it was considered that no free fat was present. Thus, in order to ascribe lecithinase activity to a sample of body fluid, in the presence of ionized calcium, it must produce turbidity which will centrifuge to the surface at least in part, and it must fail to produce turbidity in the presence of fluoride. In repeated tests, the samples of known *Cl. perfringens* lecithinase in various dilutions always conformed to these criteria.

Most of the dog fluids produced turbidity in the presence of calcium ions and lecithovitellin which could not be differentiated grossly from the turbidity produced by lecithinase. All of these fluids producing turbidity with calcium ions available, failed to produce significant turbidity in the presence of fluoride. In this respect, the activity resembled that of lecithinase. In all cases the turbidity centrifuged down entirely, however, indicating that none of the dog fluids possessed demonstrable activity. Examples of these reactions are reproduced in Table II.

TABLE II. RESULTS OF DETERMINATION OF LECITHINASE IN BODY FLUIDS

| TEST SUBSTANCE 0.5 ML. | Na F (ML.) | INCREASE IN TURBIDITY (KLETT UNITS*) | DENSITY OF PRECIPITATE RELATIVE TO SALINE |
|---|---|---|---|
| Lecithinase | 0.1 | 0 | — |
| | — | 265 | Lighter |
| Peritoneal Fluid No. 635 | 0.1 | 0 | — |
| | — | 109 | Heavier |
| Fluid No. 635 + Lecithinase 1:1 | 0.1 | 0 | — |
| | — | 130 | Heavier |

Saline added to bring tube contents to constant volume.

*Klett values for increase in turbidity over zero time corrected for turbidity of controls. The values recorded are subject to moderate error due to the flocculent nature of the turbidity developed over twenty-four hours' incubation.

Samples of dog fluid and solutions of known lecithinase were mixed and assayed for activity by these criteria. Again, although strong turbidity was produced in the absence of fluoride and with none in its presence, all such precipitates centrifuged down, indicating the lack of lecithinase activity.

Sterile egg-yolk solution, upon aging sufficiently in the refrigerator, produced a similar turbidity upon dilution with saline solution and incubation at 37° C. This has also been the experience of others.[9, 11] This precipitate was also inhibited by fluoride but centrifuged down, so it lacked the qualifications for being a product of a lecithinase reaction.

## DISCUSSION

These experimental results emphasize that the use of the lecithovitellin reaction to detect perfringens lecithinase in body fluids may lead to erroneous conclusions about the presence of such an enzyme, unless the reaction is suitably controlled. It is suggested that the partial identification of fat formation through its flotation be added to specific antitoxin inhibition to improve the specificity of the reaction. The fluoride inhibition test proved to be entirely superfluous as an additional differentiating characteristic.

A number of investigators have employed perfringens antitoxin to inhibit the lecithovitellin reaction in order to prove specificity.[1, 9] Horse antitoxin has generally been used, although Anderson and Tanturi[1] also used a normal dog serum found to be strongly inhibitory for known lecithinase and thought to contain antitoxin. However, Crook[11] and Owen and associates[12] observed that the sera of most normal horses and dogs inhibited the lecithovitellin turbidity reaction. Wensinck and Harenburg[13] reasonably concluded that the inhibitory power of normal animal serum was not caused by the possible presence of specific antitoxin therein, since such serum failed to inhibit the release of carbon dioxide by lecithinase, using the manometric technic of Zamecnik and associates,[14] although it did readily inhibit the lecithovitellin turbidity formation. Specific perfringens antitoxin inhibited both reactions of lecithinase. Therefore, one must conclude that antitoxic serum inhibition of the lecithovitellin reaction is only specific if the antiserum is diluted beyond the range of nonspecific serum inhibition. It is, of course, possible that upon further investigation it will be found that normal serum will inhibit only bacterial lecithinase in the turbidity reaction, and will differentiate true lecithinase activity from pseudoactivity of the type we found in dog fluids and in aged egg-yolk solution.

Tanturi and colleagues[2-4] also applied to some of their samples a further characterization of lecithinase based on the work of Macfarlane and Knight:[9] that of measuring the increase in acid-soluble phosphorus after incubation of the dog fluid with the substrate. It is conceivable that in body fluids, especially intestinal contents rich in digestive enzymes, there are substances other than lecithinases which will liberate fat or soluble phosphorus compounds from egg-yolk solution. Therefore, it is recommended, when perfringens lecithinase activity is sought in body fluids by means of the lecithovitellin reaction, that perfringens antitoxin inhibition using properly diluted antiserum be the basis for claiming specificity. But, because different batches of serum vary in their nonspecific inhibitory properties and because it is difficult to know when a given sample of antitoxin is diluted beyond its nonspecific range, other criteria should be applied to the system of lecithinase activity.

## SUMMARY

Body fluids from dogs with intestinal strangulation showed activity which resembled that of perfringens lecithinase in the lecithovitellin turbidity reaction. Upon applying a criterion of lecithinase activity based on its properties, it was found that none of the samples of dog fluid possessed true lecithinase activity. The criterion used was turbidity flotation after centrifugation, indicating fat formation. A fluoride-inhibition test also was tried but proved to be nondiscriminatory. It is believed that tests of this nature may be of value in fortifying the specific antitoxin inhibition of lecithinase, since the latter, through normal serum factors, can inhibit lecithinase nonspecifically over a range of dilutions.

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# AN IMPROVED CALIPER FOR MEASUREMENT OF SKINFOLD THICKNESS

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## INTRODUCTION

THE ability to quantitate relative leanness-fatness is of value to the physician in handling problems of under- and overweight and is important to the nutritionist for the evaluation of nutritional status in populations. The measurement of skinfold thickness has been found of value in this appraisal.[1] Though the relation of skinfold thickness to relative obesity has been known for some time, it is only recently, since more objective measurements such as body specific gravity and total body water have become available, that methods have begun to be quantitated and received widespread recognition.[1]

Richer[2] described in 1890 what are probably the first calipers used for the measurement of skinfold thickness in man. The literature on the use of calipers for skinfold measurements has been recently reviewed.[3]

Most of the instruments used by the various authors pivot about a single fulcrum so that with wide jaw openings the orientation of contact faces is not parallel. The size and shape of contact faces also varies. Some are flat, others are mound or ridge shaped. They may be round or rectangular in cross section, and from a few millimeters to about a centimeter in diameter. Some have corrugated contacts to ensure against slipping if the skin is moist. The method of obtaining tension is also variable. In some instances, the tension is applied by the observer's grip on the calipers. In others a spring exerts pressure constantly. In most such instances a greater tension is exerted by wider jaw openings. The scale varies in unitage and readability. Some calipers have smaller scales than the actual indicated openings due to leverage effects. In others a constant factor must be subtracted. We have seen one commercial model in which the unitage bore no logical relationship to the metric or English system of measurements. Legibility of scales is sometimes poor. Some instruments have locks so that a reading can be taken after the caliper is removed. This appears superfluous if the scale is clearly marked and well located. As a matter of fact, some discomfort to the subject might result from removing the calipers without opening the jaws.

There has been a free interchange of objectives and ideas on caliper design among those in this country currently interested in the subject. The caliper to be described was designed to have round, flat faces of a diameter and with a

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The opinions expressed in this paper are those of the authors and do not necessarily represent those of any governmental agency.

Received for publication, Feb. 26, 1954.

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tension recommended in such interchange.[4] Subsequent studies have indicated that the type of contact face might be improved. Any such modification should be entirely feasible following the basic design of the caliper.

## MATERIALS AND METHODS

*Description of Caliper.*—The caliper in its present form is illustrated in Fig. 1. There are three principal moving components: (1) a rigid frame, slide housing (*F*), and thumb grip, (2) a calibrated sliding contact piece, and (3) a scissors-like finger grip and tension-adjusting pointer (*D*). Parts 1 and 3 pivot about the connecting bolt. Parts 2 and 3 are joined through a spring (*C*) and adjustable length bolt combination (*AB*). Part 2 slides through the slide housing of part 1 to give always parallel, opposed orientation of the two identical contact surfaces (*G*). These surfaces are round and flat with a diameter of 3 mm. and an area of approximately 7 sq. mm. When the contact faces touch each other, the pointer in the slide housing (*F*) indicates zero on the scale of the slide. Lateral contact with the slide occurs only in the upper and lower portions of the slide housing to reduce friction. The tension of the spring (*C*) is adjusted with the adjustable nut and bolt combination (*AB*) at the head of the slide so that the tension is 200 Gm. when the pointer of the scissors arm (*D*) points at the tension-indicating line on the slide at *E*. A hole is present at the end of the slide at *B* so that a weight or precalibrated spring may be attached for standardization of the calipers. The present calipers were constructed primarily of dural metal, though other metals would prove equally suitable.*

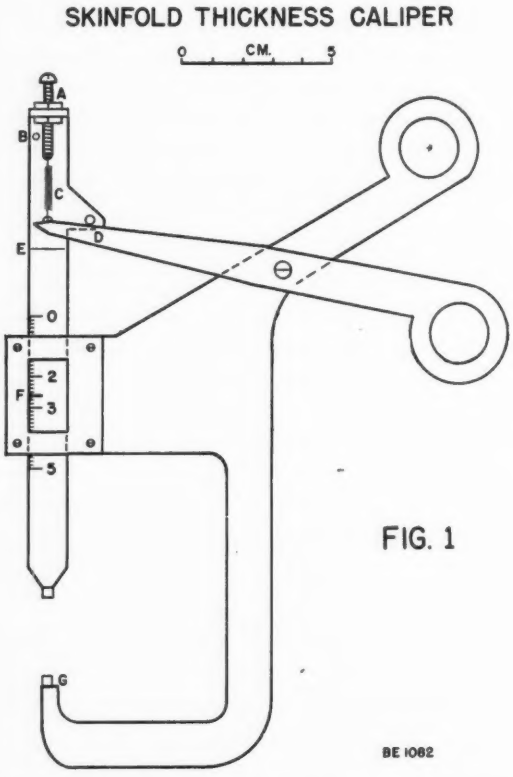


FIG. 1

*Use of the Caliper.*—The instrument is grasped in the right hand, the thumb through the upper grip hole, the fourth finger through the lower. The

*Mr. Vern Gersmehl of 3817 Warren Avenue, Cheyenne, Wyoming, constructed the present calipers according to our specifications.

body and scissors arm of the caliper are steadied with the first and second fingers. The jaws of the caliper are opened by opening the scissors mechanism. The stop on the slide catches on the pointer end of the scissors arm, causing the slide to move out. The skinfold to be measured is grasped with the thumb and index finger of the left hand. The contact faces are applied to the folded skin at least a centimeter away from the left hand. The size of bite depends on the thickness and relative tightness of the skin, generally varying from 0.5 to 6 cm. along the surface. The pointer is brought level with the tension index line on the slide and maintained there for several seconds while the entire instrument is moved back and forth slightly to overcome any tendency for friction in the slide mechanism. The skinfold thickness is read to the nearest half millimeter on the slide scale; the jaws are again opened, and the instrument removed.

The instrument is capable of measuring skinfolds up to 5 cm. in thickness at any cutaneous site. The sites of measurement in the studies of this laboratory have generally been the following: (a) a transverse skinfold one-third of the distance from the navel to the right mid-lateral line, (b) a transverse fold halfway between the right nipple and the apex of the right anterior axillary fold, (c) a longitudinal fold midway between the olecranon and the head of the humerus on the dorsal surface of the right brachium, (d) a horizontal to oblique fold below the tip of the right scapula, and (e) a vertical fold about 2 cm. above the upper margin of the right patella.

## DISCUSSION

The present type of caliper has been used in more than a thousand determinations of skinfold thickness in the sites mentioned. It has been found easy to manipulate, easy to read, and not uncomfortable to the subjects. Studies have been performed to ascertain the reproducibility of measurements and the standard deviation of a single measurement was found to be 0.0743 cm. When the instrument is properly lubricated and used, friction in the slide housing does not appear to be an important factor. A constant tension of 28.5 Gm. per cubic millimeter is applied with this instrument regardless of the jaw opening.

Keys and Brozek[5] have recently modified a Vernier slide caliper to give a constant tension of 400 Gm. through parallel, opposed, flat, rectangular faces measuring 3 by 13 mm. A shearing action occurs while the pressure is being built up with the thumb-operated drive wheel. It is uncomfortable for the operator to make a large number of measurements with this thumb-actuated device. In obese subjects the frame of the instrument may interfere with the skinfold. Two centimeters must be subtracted from each reading. We are in the process of comparing this caliper with our own in a series of subjects. If the larger, rectangular contact faces prove to be superior, the present model can be modified to this configuration.

As yet there are no universal formulas for the conversion of skinfold thicknesses as related to age and sex into corresponding figures of percentage of body fat. We have utilized the equations of Brozek and Keys[3] and Rathbun and Pace[6] to give an estimate of percentage of body fat in young men. The

derived values can be only approximate because the present caliper has slightly different specifications than that formerly used by Brozek and Keys in deriving this equation; our sites of measurement are apparently slightly different from those used by these authors; they estimated residual air volumes in the measurement of specific gravity on which the formulas are based; and the constants used in the Rathbun and Pace formula are open to question.[5] Nevertheless, estimates based on these equations have proved of value in field studies on military personnel. It is expected that more suitable prediction equations having broader practical value will become available within the next few years. Nomograms can generally be constructed to simplify the application of such formulas.

SUMMARY

A skinfold caliper has been developed which is easily manipulated with one hand, maintains a constant tension on the flat, parallel faces regardless of skinfold size, and on which readings may be readily and accurately made.

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## Erratum

In the article by Bowman, Trantham, and Caulfield, entitled "An Instrument and Method for Rapid, Dependable Determination of Freezing-Point Depression," which appeared in the February, 1954, issue of the JOURNAL, pages 310-315, the sentence beginning at the end of the eighth line on page 312 should read as follows: "Each step of the step switch is 15,000 ohms or about 1° C."

In the article by Vitale, Zamcheck, DiGiorgio, and Hegsted entitled "Effects of Aminopterin Administration on the Respiration and Morphology of the Gastrointestinal Mucosa of Rats," appearing in the April, 1954, issue of the JOURNAL, the figures in the last column of Table II on page 584 should read as follows:

14.2 ± 1.4
14.8 ± 1.8

# The Journal of Laboratory and Clinical Medicine

**The Official Publication of the Central Society for Clinical Research**

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University of Chicago School of Medicine
950 East 59th Street
Chicago 37, Illinois

PUBLISHED BY THE C. V. MOSBY COMPANY, 3207 WASHINGTON BLVD.,
ST. LOUIS 3, U. S. A.

Published monthly. Subscriptions may begin at any time.

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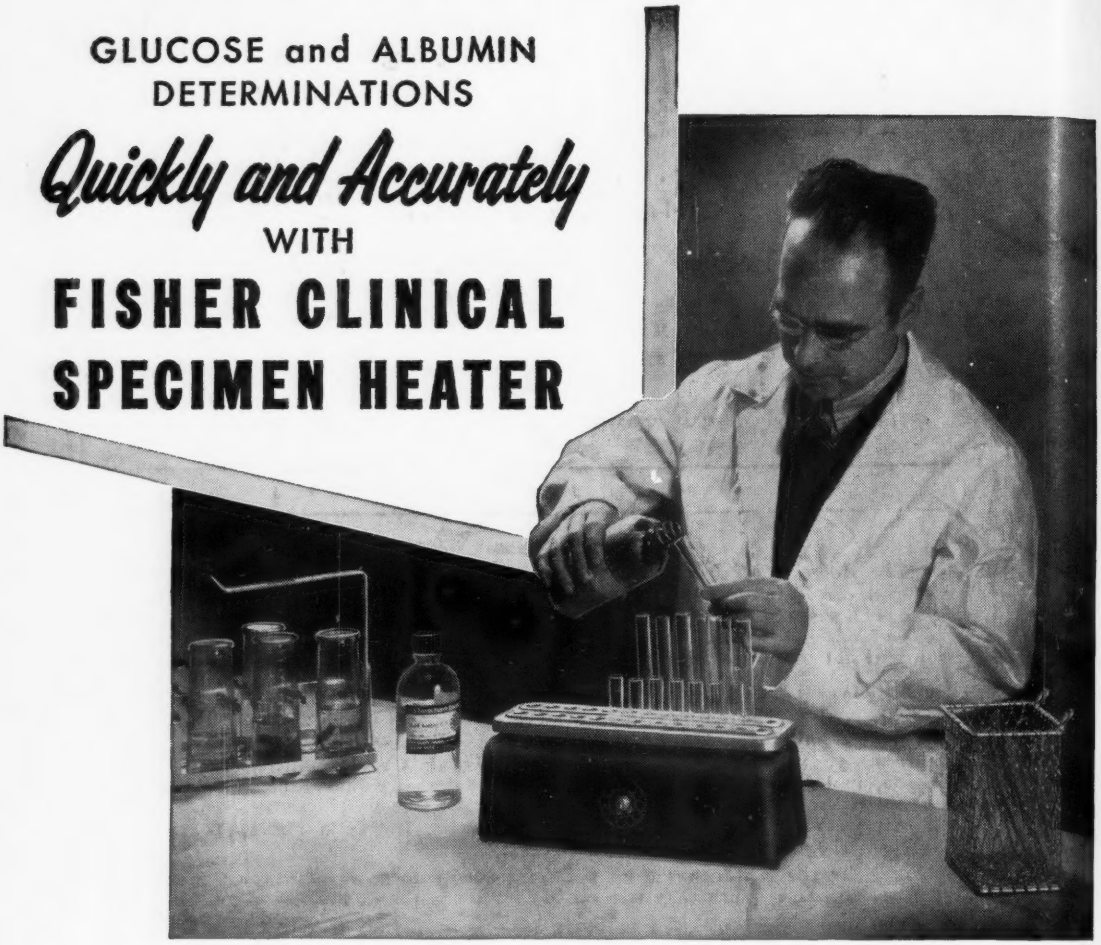

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